On the influence of the protein kinases pCKII, STN7 and STN8

on chloroplast phosphorylation networks in *Arabidopsis thaliana*

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„Remember to look up at the stars

and not down at your feet. "

Stephen Hawking 2018

Abriss

Proteinphosphorylierungen sind reversible posttranslationale Modifikationen, die oft als molekulare Schalter für biochemische Signalwege dienen. Sie haben wesentlichen Einfluss auf die Regulation der Photosynthese. Vergangene Studien zeigten, dass auch phosphorylierungsbedingte Langzeitanpassungen des plastidären Metabolismus, sowie der chloroplastidären und nukleären Genexpression auftreten können. Um derartige Mechanismen und Knotenpunkte der Signalwege besser zu verstehen, wurden im Rahmen dieser Dissertation Proteinphosphorylierungsstudien am Chloroplasten von *Arabidopsis thaliana* durchgeführt. Dafür wurden mittels Kinaseaktivitätsassays auf einem Peptid-Microarray Substrate der plastidären Caseinkinase II (pCKII) zugeordnet. Mittels dieser in vitro-Methode konnte u.A. das thylakoidäre Albino-3 (Alb3) Protein als eindeutiges pCKII-Target identifziert werden. Die exakte Phosphorylierungsstelle wurde anschließend exemplarisch für dieses Substrat mittels rekombinant erstellter Phosphostellen-Mutanten analysiert. Für die Identifikation von Targets der thylakoidären STN7- und STN8-Kinase wurde ein komparativer Massenspektrometrie-basierter in vivo Phosphoproteomik-Ansatz entwickelt. Die chloroplastidären Phosphorylierungsmuster von Wildtyp Pflanzen und den entsprechenden t-DNA Insertionsmutanten beider Kinasen wurden dafür verglichen. Mit der gewählten experimentellen Strategie wurden überwiegend stromale Substrate, insbesondere solche der chloroplastidären Genexpressionsmaschinerie entdeckt. Die Ergebnisse dieser Dissertation zeigen, dass die drei Kinasen pCKII, STN7 und STN8 Mediatoren zwischen der photosynthetischen und metabolischen Funktion, sowie der Langzeitanpassung im Chloroplasten sind.

Abstract

Protein phosphorylations are reversible posttranslational modifications (PTMs), which serve often as molecular switches for biochemical pathways. Their influence on the regulation of photosynthesis is already well known. Former studies show a further influence on long-term acclimations of the plastid metabolism and the plastid and nuclear gene expression. In order to understand such mechanisms and network nodes of the corresponding pathways were phosphorylation studies on the chloroplasts of *Arabidopsis thaliana* conducted. Kinase activity assays on a peptide microarray revealed substrates of the plastid casein kinase II (pCKII). With this in vitro-method was the thylakoid Albino-3 (Alb3) protein detected as a clear pCKII-target. Its exact phosphorylation site was analyzed via recombinant generated phosphosite mutant proteins. An in vivo mass spectrometry based comparative phosphoproteomics approach was developed for the target identification of the thylakoid STN7- und STN8-kinases. The chloroplast phosphorylation patterns of the wild-type plants compared to the respective t-DNA insertion mutants of both kinases were compared. With the chosen experimental strategy mainly stromal targets, especially such of the plastid gene expression machinery were found. The results of this thesis show that the three kinases pCKII, STN7 and STN8 are mediators between the photosynthetic and the metabolic function of the chloroplast and its long-term adaptation to prevailing light conditions.

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List of Abbreviations

1,3 DPG 1,3-bisphophoglycerate

16S .... 16 Svedberg

2D.. two-dimensional

3D.. three-dimensional

3-PG 3-phosphoglycerate

70S .... 70 Svedberg

A. thaliana .... *Arabidopsis thaliana*

A. tumefaciens.. *Agrobacterium tumefaciens*

A2B2...GAPDH protein complex consisting of 2 GAPA and 2 GAPB molecules

ABA .... abscisic acid

ABC1K.. activity of bc1-complex-like kinase

AIMS..accurate inclusion mass screening

Alb 3 .... albino 3

Arg.. arginine

ATM.. ataxia telangiectasia-mutated

ATP ....adenosine triphosphate

ATR.. ataxia telangiectasia mutated and rad3-related

CaMV 35S ....cauliflower mosaic virus 35 Svedberg

CaS .... calcium sensing protein

CEF .... cyclic electron flow

CID.. collision induced dissociation

CKII .... casein kinase II

ClpP3 .... caseinolytic protease proteolytic subunit 3

CND41 ....chloroplast nucleoid DNAbinding protein 41 kDa

CO_2 .... carbon dioxide

Col 0 .... Columbia zero ecotype

CoRR.. Co-localization for Redox Regulation

CP43 .... photosystem II reaction center protein 43 kDa

cpHsc70-2.. chloroplast heat shock protein 70-2

CSK .... chloroplast sensor kinase

CtpA .... C-terminal processing peptidase A

CTR1K... constitutive triple response kinase 1

List of abbreviations

D1 ....photosystem II reaction center protein D1

D2 ....photosystem II reaction center protein D2

Da .... Dalton

Deg .... degradation protease

DNA ....desoxyribonucleic acid

EF1B .... translation elongation factor 1B

EF-Tu .... elongation factor Tu

ESI .... electrospray ionization

ESI-MS ....electrospray ionization –mass spectrometry

ETD .... electron transfer dissociation

FDR .... false discovery rate

FLN1 .... fructokinase-like 1

FLN2 .... fructokinase-like 2

FNR... ferredoxin-NADP+ oxidoreductase

FSD2 ....iron superoxide dismutase 2

FSD3 ....iron superoxide dismutase 3

FtsH .... filamentation temperature sensitive protein

FtsZ..filamenting temperature-sensitive mutant Z

G6P glucose-6-phosphate

GAP .... glyceraldehyde 3-phosphate

GAPA ....glyceraldehyde-3-phosphate dehydrogenase protein A

GAPB ....glyceraldehyde-3-phosphate dehydrogenase protein B

GAPDH...glyceraldehyde-3-phosphate dehydrogenase

GS/GT ....glycine-serine/ glycince-threonine

GSK3 ....glycogen synthase kinase 3

GTP...guanosine triphosphate

GyrA .... DNA gyrase subunit A

GyrB .... DNA gyrase subunit B

H$_2$O .... water

H$_3$PO$_4$.. phosphoric acid

Heparin Sepharose® CL6B ....immobilized heparin on a 6% cross-linked agarose-matrix

His .... histidine

HL ....high light

HPO$_3$... meta- phosphoric acid

X

IMAC .... immobilized metal affinity chromatography

iTRAQ .... isobaric tags for relative and absolute quantitation

LC-HD-MS^E .... liquid chromatography-high definition-mass spectrometry^E

LC-MS/MS .... liquid chromatography tandem MS

LEF...linear electron flow

LHC ....light harvesting complex

Lhca3 ....light-harvesting complex 1protein 3

Lhcb2.. light harvesting complex II protein 2

LHCII ....light harvesting complex II

LTR ....long term response

m/z .... mass to charge ratio

Mbp... megabase pair

MBP... maltose-binding protein

MFP1 .... MAR-binding filament-like protein 1

mRNA ....messenger RNA

MS .... mass spectrometry

MSA.. multi stage activation

MSCS-3 .... mechanosensitive ion channel protein 3

MSH1..MutS Homolog 1

MSK4 .... Medicago sativa GSK3-like kinase 4

MurE ....Mur ligase E domain-containing protein

myc.. myelocytomatosis viral oncogene homolog

NADP$^+$.. nicotinamide adenine dinucleotide phosphate

NADPH .... hydrogen bonded reduced version of nicotinamide adenine dinucleotide phosphate

NEP...nuclear-encoded plastid RNA-polymerase

O$_2$ .... oxygen

PAPs ....PEP associated proteins

PBCP ....photosystem II core phosphatase

pCKII .... plastid casein kinase II

PEND ....plastid envelope DNA-binding protein

PEP .... plastid RNA polymerase complex

PGK1...phosphoglycerate-kinase 1

PGRL1A..proton gradient regulation 5-like A protein

Phos-tag SDS-PAGE ....phosphate-binding-tag SDS-PAGE

PKA ....protein kinase A

PolIB .... DNA polymerase gamma 1/DNA-directed DNA polymerase

PPH1...protein phosphatase 1

ppm..parts per million

PRIN2 .... plastid redox insensitive 2

psaAB .... photosystem I A and B operon

PSBH...photosystem II reaction center protein H

PSI ....photosystem one

PSII ....photosystem two

PSI-LHCI-LHCII supercomplex ....photosystem I-light harvesting complex I and II supercomplex

PTK ....plastid transcription kinase

PTM...posttranslational modification

RBCL ....large subunit of RuBisCo

RBCS ....small subunit of RuBisCo

RBCS1A...small subunit of RuBisCo protein 1A

RBCS1B...small subunit of RuBisCo protein 1B

RBCS2B...small subunit of RuBisCo protein 2B

RBCS3B...small subunit of RuBisCo protein 3B

RCA..ribulose-1,5bisphosphate-carboxylase/-oxygenase (RuBisCo) activase

RH3...DEAD box RNA helicase 3

RNA ....ribonucleic acid

RNP29 .... ribonucleoprotein particle 29

RNP31 .... 31kDa RNA binding protein

RNPs...RNA binding proteins

rpo.. RNA polymerase core, RNA polymerase

Rps7 ....chloroplast ribosomal protein S7

rRNA...ribosomal ribonucleic acid

RuBisCo .... ribulose-bisphosphate carboxylase

Rug2 .... Rugosa 2

S424A ....serine 424 alanine

SCX .... strong cation exchange chromatography

SDS-PAGE ....dodecylsulfate polyacrylamide gel electrophoresis

Ser ....serine

Ser/Thr ....serine/ threonine

SIG1 ....RNA polymerase sigma subunit 1 protein

SIG2 .... sigma factor 2

SIG3 .... sigma factor 3

SIG6 .... sigma factor 6

SiR .... sulfite reductase

SNAP33.. soluble N-ethylmaleimide-sensitive factor adaptor protein 33

SNF1 ....sucrose nonfermenting 1

STN7 ....state transition 7 kinase

STN8 ....state transition 8 kinase

stt7 .... state transition 7 kinase in Chlamydomonas reinhardtii

SWIBs .... SWI/SNF complex b domain-containing proteins

TAC.. transcriptionally active chromosome

TAC 10 ....transcriptionally active chromosome protein 10

TAC16...transcriptionally active chromosome 16 protein

TAK1 .... thylakoid associated kinase 1

TAKs ....thylakoid associated kinases

TAP38 ....thylakoid-associated phosphatase 38

TCP34 ....tetratricopeptide-containing chloroplast protein of 34 kDa

T-DNA.. transfer desoxyribonucleic acid

TIC .... translocon at the inner envelope membrane

TIC 62 ....translocon at the inner chloroplast envelope membrane protein 62

TiO$_2$ ....titanium dioxide

TiO$_2$-MOAC...titanium dioxide-metal oxide affinity chromatography

TOC..translocon at the outer envelope membrane

TOC 159......................................translocon at the outer chloroplast envelope membrane protein 159

TROL..thylakoid rhodanese like

TrxZ ....thioredoxin Z

TSP9 .... thylakoid soluble phosphoprotein 9

Tyr ....tyrosine

VIPP...vesicle-inducing protein in plastids

WHY1 .... whirly 1

WHY3 .... whirly 3

WT...wild-type

$_y$^{32}P- .... gammaphosphate with radioactive 32 phosphor

$_y{}^{33}P$- .... gammaphosphate with radioactive 33 phosphor

1   Introduction

1.1   Chloroplasts in the plant cell

Chloroplasts are in charge of photosynthesis and convert thereby sun into chemical energy (Sugiura, 1992). This process enables life for heterotrophic organisms. Chloroplasts are lenticular organelles, which exist in green algae and higher plants. Typical mesophyll cells of green leaves harbor more than 100 of such green plastids, whose mean size is about 50 µm² (Pyke & Leech, 1994). **Figure 1** illustrates a scheme of a typical chloroplast, which consists of three major subcompartments: the thylakoid lumen, the stroma and the intermembrane space between both envelope membranes. The stroma is marbled by an inner chlorophyll containing vesicular membrane system: the thylakoids, which can be arranged as stroma-lamella or grana-stacks. These thylakoids accommodate the photosynthesis, which consists of the light-dependent reaction and the Calvin-Benson cycle.
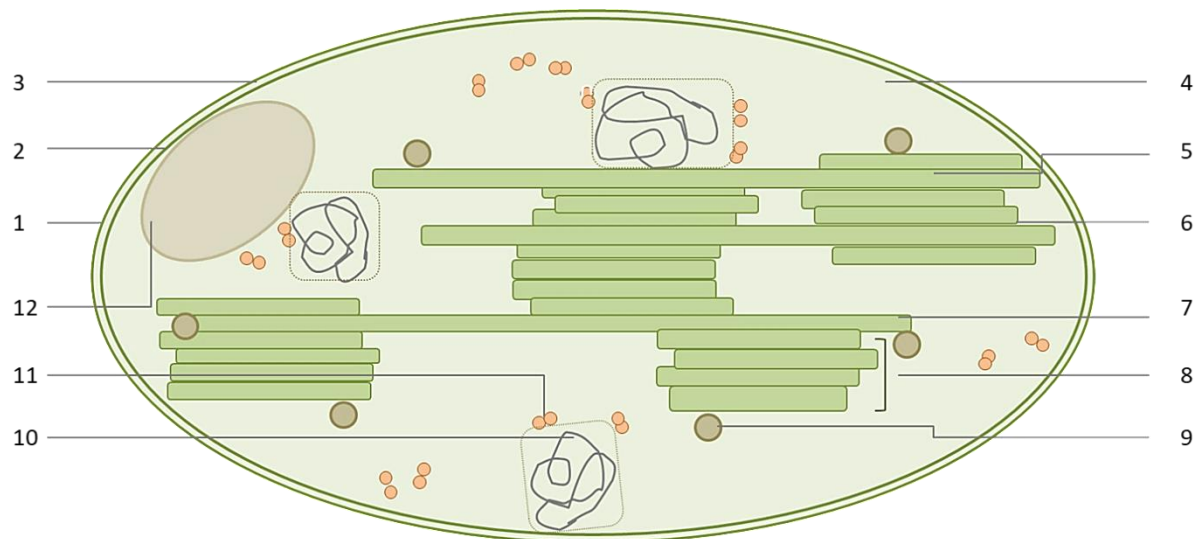
Figure 1 Scheme of a chloroplast : 1) outer envelope membrane, 2) inner envelope membrane, 3) intermembrane space, 4) stroma, 5) thylakoid lumen, 6) thylakoid membrane, 7) stromal lamella (thylakoid), 8) granum (thylakoid stacks), 9) plastoglobulus, 10) nucleoid, 11) ribosomes, 12) starch granule.

Chloroplasts originated during evolution by an event called endosymbiosis, whereby a phototrophic cyanobacterium integrated into an eukaryotic heterotrophic cell (Douglas, 1998; Margulis, 1971; McFadden, 1999). Due to gene transfer from symbiont to nucleus many chloroplast proteins are encoded in the nuclear genome and imported into the plastids post-translationally (Bock & Timmis, 2008; Martin & Herrmann, 1998). Most of these proteins accommodate N-terminal transit peptides for the targeting specificity (Agne *et al.*, 2009; Jarvis, 2008). The main import route for nucleus

encoded plastid proteins is formed by translocons at the outer envelope membrane (TOC) and the inner envelop membrane of the chloroplast (TIC) (Shi & Theg, 2013). In the stroma the N-terminal transit peptides are cleaved off from the full length proteins by a stromal processing peptidase (Richter & Lamppa, 1998; Robinson & Ellis, 1984). Finally the resulting mature proteins are integrated into the chloroplast system.

Despite this nuclear encoded plastid proteins some proteins are still encoded by the chloroplast genome, especially those with structural and enzymatic functions in photosynthesis, lipid metabolism and gene expression. They are synthesized via the plastid gene expression system, which consists of the nucleoid and ribosomes, located in the stroma (Majeran *et al.*, 2012; Yagi & Shiina, 2014). Chloroplasts contribute to the nitrogen, sulfur and carbon assimilation (Sugiura, 1992).

The thylakoid membrane shelters the light-dependent reaction of the photosynthesis. During this reaction absorb chlorophyll molecules of the light harvesting complex photons and release energy rich electrons. These electrons are passed through the so called electron transport chain and reduce nicotinamide adenine dinucleotide phosphate ($NADP^+$) to its hydrogen bonded version (NADPH). In parallel a proton gradient is built across the thylakoid membrane, which is used for the synthesis of adenosine triphosphate (ATP). The chlorophyll molecules fill their electron gaps via water (H_2O) molecules. These water molecules are split into oxygen (O_2) and protons under oxygenic photosynthesis. The NADPH and ATP molecules are light-dependent produced and become used as reduction equivalents and energy resources during the light-independent Calvin-Benson cycle for the carbon fixation in the stroma. This process starts with the reaction of carbon dioxide (CO_2) and ribulose 1,5-bisphosphate to two molecules of 3-phosphoglycerate by the function of ribulose-bisphosphate carboxylase (RuBisCo). These 3-phosphoglycerate molecules are phosphorylated by ATP to 1,3-bisphosphoglycerate which is then reduced by NADPH to glyceraldehyde 3-phosphate. Glyceraldehyde 3-phosphate (GAP) is converted to fructose 6-phosphate and thereafter to ribulose 5-phosphate, which becomes phosphorylated by ATP in order to regenerate the CO_2 acceptor ribulose 1,5-bisphosphate. The resulting GAP molecules are the starting point for the generation of long-chained carbohydrates.

1.2   Arabidopsis thaliana as a model plant

During the last four decades *Arabidopsis thaliana* (*A. thaliana*) became the model organism in plant research due to many convenient characteristics. It´s small size and simple growth requirements support growth under laboratory conditions (Somerville & Koornneef, 2002). It´s short generation time with a life cycle of 8 weeks, large number of offspring and it´s self- pollination allowing an easy

and fast crossing (Arabidopsis Genome Initiative, 2000; Somerville & Koornneef, 2002). It´s small genome of 125 megabase pairs (Mbp) with 25498 genes organized in 5 chromosomes is completely sequenced since the year 2000, which eases genomic and post-genomic research (Arabidopsis Genome Initiative, 2000; Meinke, 1998). This small genome encodes proteins of roughly 11000 families (Arabidopsis Genome Initiative, 2000). Pictures of soil grown *Arabidopsis thaliana* plants and their taxonomy are presented in **Figure 2**. Nowadays a plethora of shotgun proteomic approaches is well established, enabling deep proteome analyses, confirmation of gene models, expression levels and posttranslational modifications under varying situations (Wienkoop *et al.*, 2010). *Arabidopsis thaliana* can be transfected by *Agrobacterium tumefaciens (A. tumefaciens)* (Chilton *et al.*, 1977; Krysan *et al.*, 1999). The resulting transfer deoxyribonucleic acids (T-DNA) insertions in the nuclear deoxyribonucleic acid (DNA) disrupt genes, which results in a knockout of the specific gene expression. This method is broadly used for studying gene functions by comparative analyses between such mutants and their respective wild-type (WT) plants.

scientific classification	
Kingdom	Plantae
Clade	Angiosperms, Eudicots
Order	Brassicales
Family	Brassicaceae
Genus	Arabidopsis
Species	Arabidopsis thaliana
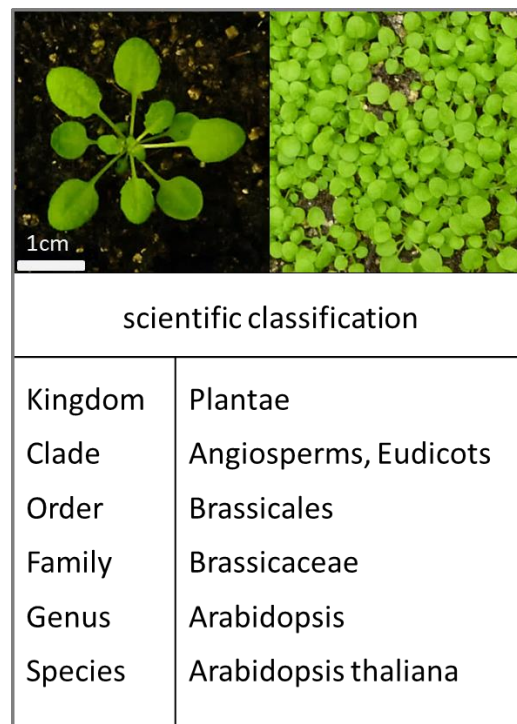
Figure 2 *Arabidopsis thaliana* : Depicted are soil grown *Arabidopsis thaliana* Columbia 0 (Col 0) plants (4 weeks 8 h light/ 16 h dark, 150µmol·m-2·s-1) as single plants (left picture) and as bulky plant populations (right picture). The scientific classification is presented below.

Due to all described benefits of this model plant and well established genomic, transcriptomic, proteomic and metabolomic methods it is nowadays possible to look with a systems biology view onto plant biochemistry.

1.3   Protein phosphorylation

1.3.1   Protein phosphorylation as posttranslational modification

Posttranslational modifications (PTMs) change protein structures covalently, whereby proteomes reach higher diversity levels (Walsh *et al.*, 2005). The addition of a phosphate group to a hydroxyl-containing amino acid, e.g. serine (Ser), threonine (Thr) or tyrosine (Tyr), or to basic residues like arginine (Arg) and histidine (His) is thereby one of the most common reversible PTMs, caused by kinase and phosphatase counteraction (Champion *et al.*, 2004; Elsholz *et al.*, 2012). **Figure 3** presents the chemical formula of the protein phosphorylation reaction. The addition of such a negatively charged moiety induces ionic interactions, which can result in changes of the protein conformation and interaction partners (Mann *et al.*, 2002). This may lead furthermore to alterations of certain catalytic processes, signal transmission and subcellular localizations (Cohen, 2002; Walsh *et al.*, 2005). Responses to internal and external stimuli can be fast relayed by fine-tuned phosphorylation cascades, where certain phosphorylation events are connected in series forming a signal transfer. This PTM is the method of choice for a rapid activation of transcriptional and translational factors (Karin & Hunter, 1995). Protein phosphorylation networks appear very complex due to the fact that a protein can be phosphorylated by several kinases at multiple sites and that these phosphorylations can be withdrawn by phosphatase activity. Plant genomes encode twice as much kinases compared to mammalian genomes underlining their significance in signal transduction of these photoautotrophic organisms (Zulawski *et al.*, 2013). In *Arabidopsis thaliana* 1052 protein kinases and 162 phosphatases were found, which regulate diverse biochemical pathways (Wang *et al.*, 2013).
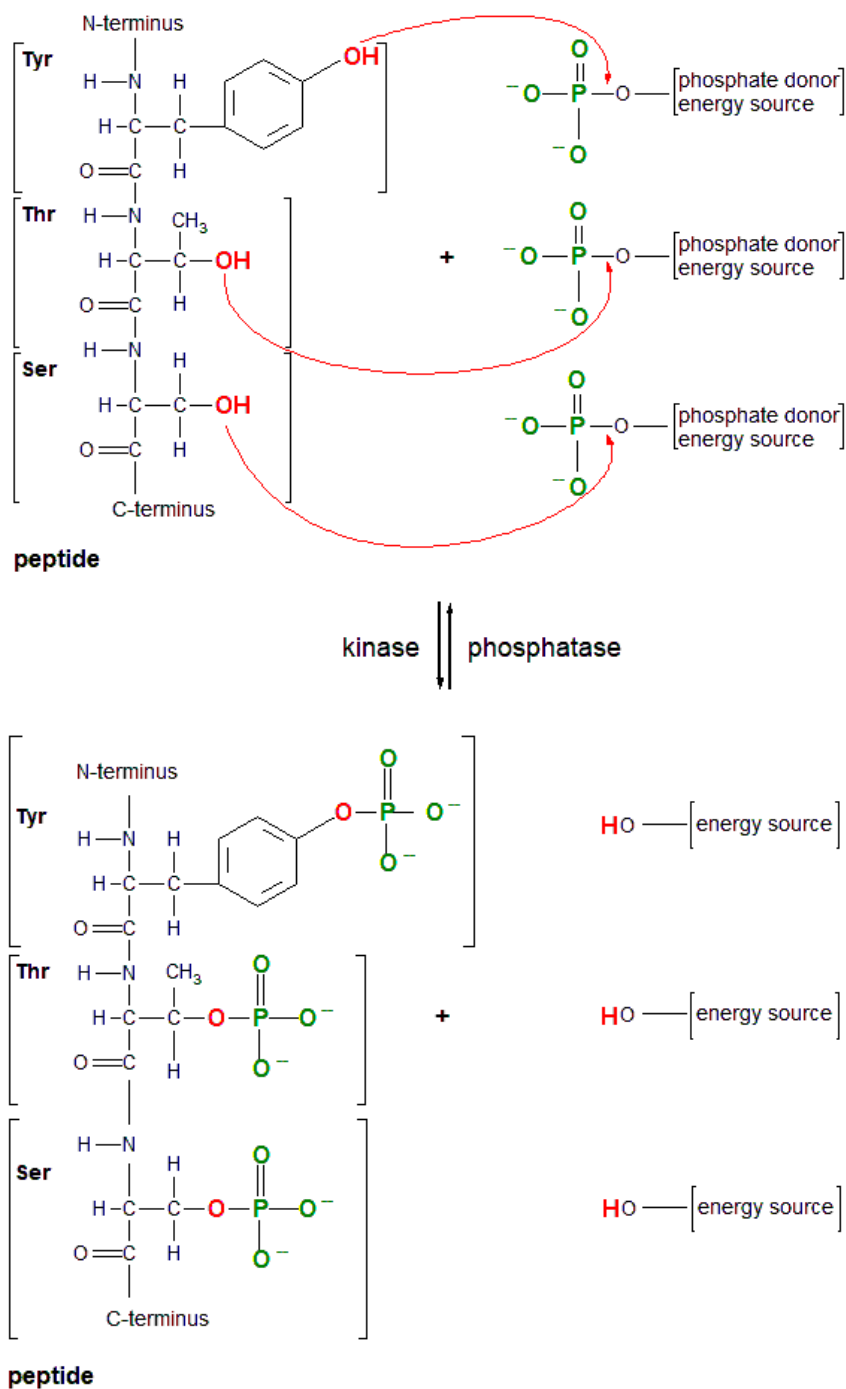
Figure 3 The protein phosphorylation reaction: This graphic illustrates the chemical formula of typical protein phosphorylations. Thereby the phosphate-group donor is presented as an energy rich compound, which can be adenosine triphosphate (ATP) or guanosine triphosphate (GTP) amongst others. A section of the protein sequence consisting of the amino acids: serine, threonine and tyrosine is depicted as reactant. Phosphorylatable hydroxyl groups are marked in red and the phosphate group is shown in green characters. In this example the nucleophile addition of phospho-groups to the hydroxyl groups of the phosphorylatable amino acids is visualized. Such phospho group additions are based on covalent phosphoric acid diester linkages.

1.3.2   Chloroplast protein phosphorylation

More than 1800 plastid proteins are known so far in *Arabidopsis thaliana* (Baginsky, 2016). Thereof over 800 proteins are detected by mass spectrometry (MS) analyses as phosphoproteins, comprising of more than 905 phosphosites (Arsova & Schulze, 2012; Durek *et al.*, 2010). Most of these phosphoproteins are functionally involved in energy conversion pathways, metabolic processes or the gene expression system (Schönberg & Baginsky, 2012). In the review of (Schönberg & Baginsky, 2012) an overview of the chloroplast protein phosphorylation network is published. This article is included in chapter 2.1 of this thesis. The article figure in chapter 2.1.2 illustrates the chloroplast phosphorylation network with the so far known kinases depicted in orange. The stromal plastid casein kinase II (pCKII) and chloroplast sensor kinase (CSK) act mainly on the plastid gene expression system. In contrast the thylakoid associated kinases state transition 7 kinase (STN7) and state transition 8 kinase (STN8) act predominantly on photosynthesis related proteins, which are involved in the energy conversion process. Their counteracting thylakoid-associated phosphatase (TAP38) and photosystem II (PSII) core phosphatase (PBCP) are depicted in blue. In the following chapters the activity of pCKII, STN7 and STN8 will be described. In addition the glycogen synthase kinase 3 (GSK3), which modulates stress tolerance and carbohydrate metabolism in plastids, will be presented (Kempa *et al.*, 2007).

1.3.2.1 Protein phosphorylations involved in energy conversion at the thylakoids

Chloroplasts are in charge of the photosynthesis, which is composed of the light-dependent reaction and its coupled Calvin-Benson cycle. During evolution this energy conversion pathway got adjusted via fine tuning regulations for a better efficiency (Allahverdiyeva *et al.*, 2015; Goldschmidt-Clermont & Bassi, 2015).

One of the most prominent and well studied example of such regulations is the process of state transitions on the thylakoid membrane (Allen *et al.*, 1981; Bennett, 1977). This process adjusts the ATP level to cellular demands (Lemeille & Rochaix, 2010). A higher excitation level of photosystem one (PSI) and a reduced plastoquinone pool lead thereby to the activation of the light harvesting complex II (LHCII) kinase (Allen *et al.*, 1981; Vener *et al.*, 1997; Zito *et al.*, 1999). This kinase phosphorylates the mobile part of LHCII and empowers its movement to PSI, leading to a photosystem I-light harvesting complex I and II supercomplex (PSI-LHCI-LHCII supercomplex) under the so called state 2 (Allen, 1992; Lemeille & Rochaix, 2010; Longoni *et al.*, 2015). Thereby excitation energy is balanced and the redox poise is restored. The responsible kinase was first identified in a

genetic screen in *Chlamydomonas reinhardtii, where the so called state transition kinase 7 (stt7)* mutant was blocked in state 1 and failed to phosphorylate LHCII under state 2 conditions (Fleischmann *et al.*, 1999). The Stt7 ortholog in *Arabidopsis thaliana* was named STN7 (Bellafiore *et al.*, 2005; Bonardi *et al.*, 2005; Vainonen *et al.*, 2005). TAP38, i.e. protein phosphatase 1 (PPH1), counteracts STN7 activity as phosphatase, supporting a change backwards to state 1, once the plastoquinone pool is oxidized and STN7 is inactive (Pribil *et al.*, 2010; Shapiguzov *et al.*, 2010). State transitions balance PSI and photosystem II (PSII) excitation energy in short term.

In long term is rather an adjustment of the photosystem stoichiometry to prevailing light conditions occurring, see chapter 2.1.2 (Depège *et al.*, 2003; Pfannschmidt, 2003). STN7 has due to significant sequence similarity a homologue, called STN8 (Bonardi *et al.*, 2005; Vainonen *et al.*, 2005). Both kinases are involved in the long term response (LTR), which connects their activity with plastid und nuclear gene expression (Bonardi *et al.*, 2005; Bräutigam *et al.*, 2009; Pesaresi *et al.*, 2009). A reduced plastoquinone pool triggers this LTR and causes a transcriptional upregulation of the plastid-encoded photosystem I A and B (psaAB) operon (Bräutigam *et al.*, 2009). Furthermore is the accumulation of the nucleus-encoded photosystem I light-harvesting complex gene 3 (Lhca3) protein raised (Pesaresi *et al.*, 2009). And alterations in the plastid metabolome, i.e. a higher accumulation rate of transitory starch and a restructuring of the thylakoid membrane become apparent (Baginsky, 2016; Pfannschmidt, 2010). It remains elusive whether STN7 is directly involved in this LTR process, e.g. by phosphorylating components of the chloroplast gene expression machinery or if this influence is a rather indirect one via an altered redox homeostasis or variations in the metabolic state (Bräutigam *et al.*, 2009; Pfannschmidt, 2010). So far no comparative phosphoproteomics study identified STN7 targets belonging to the transcriptional core of the plastid RNA polymerase complex (PEP), which might be due to its low abundance compared to high concentrated photosynthetic proteins (Pfalz & Pfannschmidt, 2013). TAC 16 a more loosely associated component of the transcriptionally active chromosome (TAC), is identified as clear STN7 target in a comparative phosphoproteomics analysis (Ingelsson & Vener, 2012). It associates the TAC complex with the thylakoid membrane, but is not belonging to the transcriptional PEP core (Ingelsson & Vener, 2012; Majeran *et al.*, 2012; Pfalz & Pfannschmidt, 2013). STN7 and STN8 are thylakoid associated serine/threonine kinases. They consist of three structural parts, namely the stroma exposed catalytic domain, a thylakoid transmembrane domain and their lumenal N-terminus (Lemeille *et al.*, 2009). Beside their homology and similar protein structure differ the main target spectra of these kinases.

STN8 is the kinase for the phosphorylation of PSII core subunits, e.g. the photosystem II reaction center proteins 43 (CP43), D1 and D2 (Bonardi *et al.*, 2005; Vainonen *et al.*, 2005; Vainonen *et al.*,

2008). Upon photodamage becomes D1 phosphorylated, which enables its rapid turnover by the unpacking of PSII supercomplexes and initiates the migration of PSII monomers from grana to stroma lamellae (Adam *et al.*, 2006; Fristedt *et al.*, 2009; Khatoon *et al.*, 2009; Tikkanen *et al.*, 2008). D1 gets dephosphorylated at this stromal thylakoid sites by PBCP and degraded by filamentation temperature sensitive (FtsH) and degradation (Deg) proteases (Kato & Sakamoto, 2014; Wunder *et al.*, 2013). A new D1 subunit which is de novo synthesized at the plastid 70S ribosome subunit and processed by C-terminal processing peptidase A (CtpA) peptidase is then inserted into the truncated PSII complex (Puthiyaveetil *et al.*, 2014). Comparative phosphoproteomics revealed e.g. the proton gradient regulation 5-like A protein (PGRL1A) as a further STN8 substrate, which causes once phosphorylated a faster switch from cyclic to linear electron flow upon the onset of illumination (Reiland *et al.*, 2011). STN8 phosphorylates furthermore PSII reaction center protein H (PSBH) and the Calcium sensing protein (CaS), which maybe connects its activity to Calcium signalling (Vainonen *et al.*, 2008).

For STN7 and its paralog STN8 is a substrate overlap reported due to residual phosphorylations of LHCII and PSII components in the single mutants (Bonardi *et al.*, 2005; Samol *et al.*, 2012; Vainonen *et al.*, 2008). These phosphorylations are completely lacking in the double mutant (Rochaix, 2014). Like STN7, also STN8 is redox sensitive and active under a reduced plastoquinone pool (Puthiyaveetil, 2011; Rochaix, 2011). Beyond that is STN8 in contrast to STN7 also active under high light (HL) (Puthiyaveetil, 2011). Samol et al. 2012 published the STN8 antagonist, which dephosphorylates PSII core components: the PBCP (Samol *et al.*, 2012).

In addition to these two kinases the thylakoid associated kinase 1 (TAK1) is known to act on thylakoid proteins (Pesaresi *et al.*, 2011; Snyders & Kohorn, 1999). Antisense TAK1 expression leads to a lower level of light harvesting complex (LHC) protein phosphorylation (Depège *et al.*, 2003). Once this kinase is inactivated a retardation in their growth and bleaching is observed in *Arabidopsis thaliana (Depège et al., 2003; Snyders & Kohorn, 2001)*.

1.3.2.2 Stromal protein phosphorylations involved in energy conversion

Phosphorylation events of some Calvin cycle proteins are already known, but most of the responsible kinases remain still elusive (Baginsky, 2016). Phosphorylation affects e.g. ribulose-1,5 bisphosphate-carboxylase/-oxygenase activase (RCA) at the N-terminal Thr-78, which prevents the initial activation of RuBisCo and obviates further steps of the Calvin-Benson cycle (Boex-Fontvieille *et al.*, 2014; Stotz *et al.*, 2011; van de Loo & Salvucci, 1996).

Subunits of RuBisCo itself are phosphorylation targets aswell. Thereby are the large (RBCL) and small subunits (RBCS) of RuBisCo affected (Reiland *et al.*, 2011; Roitinger *et al.*, 2015; Umezawa *et al.*, 2013). Especially under stress conditions is the phosphorylation of the small subunit proteins RBCS1A, RBCS1B, RBCS2B and RBCS3B detected (Bhaskara *et al.*, 2017; Umezawa *et al.*, 2013). These substrates are targets for a kinase, which becomes upregulated in its activity under dehydration or abscisic acid (ABA) treatment (Bhaskara *et al.*, 2017; Umezawa *et al.*, 2013). ABA is as a phytohormone a regulator of seed germination, stomatal opening and abiotic stress responses (Umezawa *et al.*, 2013). The sucrose nonfermenting 1 (SNF1)-related protein kinase 2 is suggested to be responsible for linking such stress responses with the metabolic signaling via its activity on downstream targets (Fragoso *et al.*, 2009; Halford & Hey, 2009; Shin *et al.*, 2007).

The phosphoglycerate-kinase 1 (PGK1) is a further Calvin-Benson cycle substrate identified in phosphoproteomics surveys (Bhaskara *et al.*, 2017; Reiland *et al.*, 2009; Reiland *et al.*, 2011; Roitinger *et al.*, 2015; Songyang *et al.*, 1996; Umezawa *et al.*, 2013; Yang *et al.*, 2013). Its phosphorylation status is upregulated under drought stress and ethylene treatment (Bhaskara *et al.*, 2017; Yang *et al.*, 2013). PGK1 maintains the photosynthetic capacity by phosphorylating 3-phosphoglycerate (3-PG) to 1,3-bisphosphoglycerate (1,3-DPG), which becomes thereon reduced by NADPH to GAP. It controls by its activity the starch accumulation and might be itself controlled in its activity by the identified serine 81 phosphorylation site (Umezawa *et al.*, 2013). Ethylene is known to regulate cellular processes and stress responses as a phytohormone. In this ethylene dependent signal transductions are protein kinases key players, e.g. the constitutive triple response kinase 1 (CTR1K), which is located at the endoplasmic reticulum membrane (Yang *et al.*, 2013).

Also the A and B subunits of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are found as phosphoproteins. This enzyme is in its active form a A2B2 heterotetramer and uses NADH or NADPH to reduce 1,3 bisphosphoglycerate resulting in GAP molecules. This GAP molecules are utilized as metabolites in the gluconeogenesis. Under nitrogen starvation and nitrate resupply were glyceraldehyde-3-phosphate dehydrogenase protein B (GAPB) molecules detected with an upregulated phosphorylation status (Engelsberger & Schulze, 2012). External nitrate supply regulates in general the gene expression of genes involved in nitrate transport, reduction pathways and carbohydrate metabolism (Engelsberger & Schulze, 2012; Gutiérrez *et al.*, 2007). Thereby the production of organic acids, which are necessary for the inorganic nitrogen assimilation, is coordinated (Gutiérrez *et al.*, 2007). In further large scale phosphoproteomics analyses also glyceraldehyde-3-phosphate dehydrogenase protein A (GAPA), the other subunit of GAPDH, was

found as a phosphoprotein. This finding strongly hints on a regulation circuit of GAPDH by kinase activities (Roitinger *et al.*, 2015; Umezawa *et al.*, 2013).

Another established phosphotarget functioning in the energy conversion process is the ß-subunit of the ATP synthase (ATPB) (Reiland *et al.*, 2009). This ATP synthase switches its catalytic activity from ATP synthesis to ATP hydrolysis once the electrochemical gradient across the membrane collapses. In vitro and in vivo experiments show that the responsible kinase is pCKII, which preferentially phosphorylates this protein in the dark (Kanekatsu *et al.*, 1998; Reiland *et al.*, 2009). The resulting phosphorylation is shown to cause an interaction with 14-3-3 proteins, which prevents its molecular rotation and inhibits the synthase activity in order to save ATP (Bunney *et al.*, 2001).

The GAP molecules generated by the Calvin-Benson cycle serve as metabolites in the gluconeogenesis. A part of the resulting glucose molecules are used for starch synthesis downstream of the reductive pentose phosphate pathway in the chloroplasts. Starch is composed of linear amylose and branched amylopectin molecules. It is arranged into granules and serves as the main carbohydrate store in plants. Immunofluorescense microscopy studies suggest that *Medicago sativa* GSK3-like kinase 4 (MSK4) is associated with plastid starch granules (Kempa *et al.*, 2007). This kinase is shown to connect stress signaling with carbon metabolism. Under high-salinity conditions the MSK4 in vivo kinase activity is rapidly induced (Kempa et al. 2007). Transgenic *Arabidopsis thaliana overexpressor* plants with myelocytomatosis viral oncogene homolog (myc)-tagged MSK4 under the control of cauliflower mosaic virus 35 Svedberg (CaMV 35S) promoter show upon exposure to salt stress a higher survival rate compared to WT and vector control plants (Kempa *et al.*, 2007). They contain also higher levels of the energy molecules starch, maltose, glucose and glucose-6-phosphate (G6P). Its *Arabidopsis thaliana* orthologue, the Shaggy-like kinase 1 (AtK-1), is already identified (Jonak *et al.*, 1995). This GSK3-like kinase is operating downstream the Calvin-Benson cycle, which demonstrates the multitude of metabolic regulations via protein phosphorylations.

Beside these kinases participate the activity of bc1-complex-like kinases (ABC1K), which are located at the plastoglobuli, in the regulation of photosynthesis. This regulation occurs through an influence on the prenylquinone accumulation including tocopherol, phylloquinone and plastoquinone synthesis (Lundquist *et al.*, 2012; Lundquist *et al.*, 2013; Martinis *et al.*, 2014). These examples demonstrate that phosphorylations occur on a broad variety of proteins.

1.3.2.3 Chloroplast kinases functioning in gene expression

The redox state of certain chemical components is often a key sensor for kinase activities. This is valid also for the pCKII, a serine/ threonine (Ser/Thr) kinase, which is inactivated by the reduction of certain cysteine residues via reduced gluthatione under high light (Baginsky *et al.*, 1999; Schweer *et al.*, 2010; Türkeri *et al.*, 2012). It was shown that this kinase phosphorylates many proteins involved in chloroplast gene expression, e.g. RNA binding proteins (RNPs), sigma factors and components of the transcriptionally active chromosome (TAC) (Kanekatsu *et al.*, 1993; Ogrzewalla *et al.*, 2002; Reiland *et al.*, 2009). For this reason it was formerly termed plastid transcription kinase (PTK) (Baginsky *et al.*, 1997). The addition of negatively charged phosphate groups lead to looser connections between such proteins and negatively charged nucleic acids, which represses transcription unspecifically (Türkeri *et al.*, 2012). Furthermore becomes a translational block released by the phosphorylation of RNPs once more translation is necessary (Baginsky *et al.*, 1999; Loza-Tavera *et al.*, 2006). The transcriptional activity is increased by the inactivation of pCKII, which results in a lower phosphorylation rate of the plastid encoded RNA polymerase subunits (Baena-González *et al.*, 2001). This illustrates that plastid redox homeostasis becomes linked with gene expression by the activity of pCKII. STN7 itself is assumed to be a substrate of pCKII, which might be a further regulatory link between genetic and photosynthetic pathways (Reiland *et al.*, 2009). The chloroplast sensor kinase CSK, which resembles prokaryotic two component sensor kinases, is suspected to form with pCKII and the RNA polymerase sigma subunit 1 protein (SIG1) a redox regulated regulon. This regulon is suspected to control plastid transcription activity, i.e. the expression of genes involved in photosynthesis (Puthiyaveetil *et al.*, 2012). Furthermore might STN7 and STN8 influence the plastid and nuclear gene expression by the LTR (Bräutigam *et al.*, 2009; DalCorso *et al.*, 2008; Pesaresi *et al.*, 2009). But substrates which hint on such a direct interaction were not yet found. Nevertheless is a direct interaction of the STN7 and STN8 kinase possible, for example by the phosphorylation of gene expression enzymes. Especially enzymes, which act in the transcription or translation process may serve as regulators in the LTR. Transcription in plastids of higher plants is performed by two RNA polymerases, i.e. the plastid-encoded- (PEP) and the nuclear-encoded plastid RNA polymerase (NEP) (Kühn *et al.*, 2007; Liere & Maliga, 1999). Translation is performed at the plastid ribosomes. Phosphorylation of gene expression components can result in the alteration of the proteome as an adaptation to prevailing light conditions. The nucleoid including the transcriptionally active chromosome is depicted in Figure 4. Protein components with known phosphorylation sites are marked in red. Apparently no component of the bacterial evolved RNA polymerase (rpo) core domain is found to be phosphorylated so far. All rpo components are exclusively encoded by the chloroplast genome. This indicates a substrate tendency for nuclear encoded plastid proteins of the nuclear

encoded kinases, which belong to the host plant genome from an evolutionary point of view.
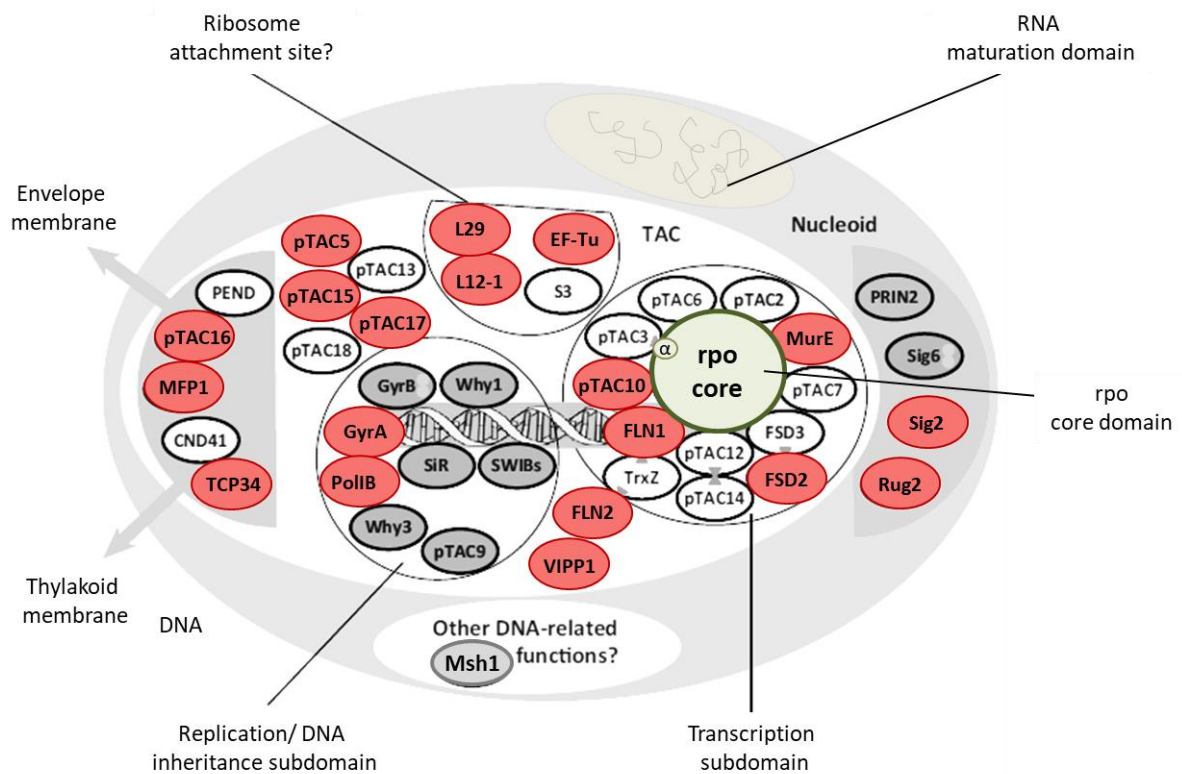
Figure 4 The nucleoid : Depicted is a modified version of the nucleoid and transcriptionally active chromosome (TAC) map, which is presented by Pfalz and Pfannschmidt 2013 in figure 1. The distribution of subdomains between TACs and nucleoids is labeled. Phosphorylated *Arabidopsis thaliana* proteins are shown as red ovals. Proteins where no corresponding phosphopeptide is detected by mass spectrometry based phosphoproteomics are illustrated in the grey scale colour code. The RNA polymerase (rpo) core is shown as a pale-green circle. It is the only compartment of the nucleoid, where no subcomponent protein is found to be phosphorylated so far. Abbreviations are similar to those used by Pfalz and Pfannschmidt 2013: chloroplast nucleoid DNAbinding protein (CND41); plastid envelope DNA-binding protein (PEND); elongation factor Tu (EF-Tu); fructokinase-like-1 (PAP6) (FLN1; fructokinase-like-2 (FLN2); iron superoxide dismutase 2 (PAP9) (FSD2); iron superoxide dismutase 3 (FSD3); DNA gyrase subunit A (GyrA); DNA gyrase subunit B (GyrB); MAR-binding filament-like protein 1 (MFP1); Mur ligase E domain-containing protein (PAP11) (MurE); MutS Homolog 1 (MSH1); DNA polymerase gamma 1/DNA-directed DNA polymerase (PolIB); plastid redox insensitive 2 (PRIN2); Rugosa2 (Rug2); sigma factor 2 (SIG2); sigma factor 6 (SIG6); sulfite reductase (SiR); SWI/SNF complex b domain-containing proteins (SWIBs); tetratricopeptide-containing chloroplast protein of 34 kDa (TCP34); thioredoxin Z (PAP10) (TrxZ); vesicle-inducing protein in plastids (VIPP); whirly 1 (WHY1); whirly 3(WHY3).

1.3.3   Detection of protein phosphorylation sites

For sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)- and Western Blot- based procedures many antibodies were developed, which recognize phosphoserines or phosphothreonines or phosphotyrosines as part of the epitope (Allahverdiyeva *et al.*, 2015; Yan *et al.*, 1999). Such antibodies show a high degree of cross reactivities with the other phosphoamino acid types, which hinders clear cut conclusions on phosphosites (Thiele *et al.*, 2009). This problem can be circumvented by using

phosphosite specific antibodies, which are trained on the protein of interest (Yan *et al.*, 1999). Another possibility of SDS-PAGE based phosphoprotein detections is the so called phosphate-binding-tag SDS-PAGE (Phos-tag SDS-PAGE) (Kinoshita *et al.*, 2006; Kinoshita & Kinoshita-Kikuta, 2011). This phosphate-binding tag is chemically a 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olatodimanganesium(II)-complex, which copolymerizes with the acrylamide matrix and forms complex formations with the phosphate groups of the proteins (Kinoshita *et al.*, 2009). This results in a slower migration of the phosphorylated proteins compared to their non-phosphorylated counterparts during electrophoresis leading to a mobility shift of distinct bands. Many improvements of this method were published, which work the best while analyzing purified proteins (Kinoshita *et al.*, 2012, 2015). Beside this is the usage of the phosphoprotein fluorescence stain Pro-Q Diamond® (Invitrogen™) on a SDS-PAGE after electrophoresis a possibility to distinguish between the phosphorylation status of different samples.

Kinase activity assays conducted with gammaphosphate radioactive ^{33}phosphor- ($_\gamma^{33}$P-) or gammaphosphate radioactive ^{32}phosphor- ($_\gamma^{32}$P-) ATP in conjunction with phosphorimaging are a further possibility to visualize phosphorylation reactions on proteins. Such kinase activity assays can be performed in batch mode with the protein separation afterwards via SDS-PAGE or on microarrays (Thiele *et al.*, 2009). Such microarrays allow the identification of kinase substrate relationships by the "wells without walls" principle (Pérez-Bercoff *et al.*, 2014; Schutkowski *et al.*, 2004). These high throughput methods allow a parallel analysis of many peptides or proteins immobilized on glass slides (Hansen *et al.*, 2017; Thiele *et al.*, 2009; Uttamchandani & Yao, 2017). Such miniaturized enzyme assays benefit of a high sensitivity due to improved signal to noise ratios, short diffusion distances and decreased reaction times (Cretich *et al.*, 2014). The ease of peptide synthesis and the long term stability of peptides allow a broad scientific usage of such high throughput multiplex analyses (Henkel *et al.*, 2016; Sobek *et al.*, 2006; Thiele *et al.*, 2009). By incubating active kinase preparations on such microarrays and visualizing their phosphorylation signals on the peptides conclusions on kinase substrate relationships can be made. A similar approach without the need for immobilization of the peptides is the kinase client (KiC) assay, which is a combination of kinase activity assays on peptides in batch mode and a MS identification of the resulting phosphopeptides afterwards (Huang & Thelen, 2012). Counteracting phosphatase and protease activities are typically blocked during such enzyme incubations, which improves higher phosphorylation rates, especially while analyzing kinase activities.

Comprehensive phosphorylation analyses cover the identification of the phosphoprotein, phosphopeptide, phosphorylation site and quantitation (Mann *et al.*, 2002). Many mass

spectrometry approaches are developed for this purpose, whereof most of them are nowadays performing in high resolution with the ability of detecting ions in femtomolar range, the improved mass accuracy till a few parts per million (ppm) for the peptide and the acceleration of duty cycles (Reiland *et al.*, 2011). Phosphopeptides are in general hard to detect via mass spectrometry due their negative charge, which impedes their detection in the positive ion modes, and their hydrophilic nature, which hinders proper binding to the columns (Mann *et al.*, 2002). Most phosphorylation events occur at low abundance, which means that only a part of the protein entity is affected. This results in peaks of low intensity, especially when the non-phosphorylated peptide is present leading to ionic suppression (Mann *et al.*, 2002). This low stoichiometry necessitates enrichment strategies of phosphopeptides as prerequisites for sufficient MS detections (Linding *et al.*, 2007). Roughly a half of the plant phosphoproteomics studies used titanium dioxide (TiO_2) for phosphopeptide enrichment and the other half a diversity of immobilized metal affinity chromatography (IMAC) strategies (van Wijk *et al.*, 2014). Reliable phosphorylation studies via MS need a robust sample preparation procedure and high accuracy MS detections. In liquid chromatography tandem MS (LC-MS/MS) approaches is the peptide identification capacity limited by the chromatographic performance of the LC-system, which is determined by its peak capacity and separation efficiency. Furthermore are the MS-scan rate and sensitivity important parameters for reliable fragment detections over a wide dynamic range (Macek *et al.*, 2009).

A robust data interpretation protocol completes the MS analysis pipeline. Especially on the level of peptide and phosphorylation site identification many false assignments occur (Baginsky, 2016). Qualitative and significant mappings are based on high mass accuracies for peptide identifications, low false discovery rates (FDR) on the entire data set, and a low number of degrees of freedom for the database matches (Lu *et al.*, 2015; Rochaix, 2014).

The ratio of MS- detected phosphorylated serine, threonine and tyrosine differs highly among the existing studies. Especially the extent of tyrosine phosphorylation is strongly debated in the community (Lu *et al.*, 2015). The $_{phospho}$serines and $_{phospho}$threonines show a predominant loss of the mass to charge ratio (m/z) of 98Da for phosphoric acid (H_3PO_4) due to ß-elimination and a loss of m/z of 80Da for meta- phosphoric acid (HPO_3), which differentiates them from $_{phospho}$tyrosines (Mann 2002). Many $_{phospho}$tyrosine MS detections are conducted with MSA approaches in order to elevate fragment ion intensities for the detection of the $_{phospho}$tyrosine specific immonium ion (m/z= 216,0426). Also arginine and histidine phosphorylations don´t get analyzed so often, which is on the one hand due to technical constraints and on the other one due to their lower occurrence in comparison to $_{phospho}$serines and $_{phospho}$threonines (van Wijk *et al.*, 2014).

1.4   Aims of this work

MS phosphoproteomic approaches revealed many chloroplast phosphorylation sites in *Arabidopsis thaliana* during the last decade (Schönberg & Baginsky, 2012). But for most of these phosphoproteins information about the responsible kinase is lacking. In order to unravel such kinase substrate relationships the creation of a peptide microarray as an in vitro high throughput method is aimed. The chloroplast casein kinase II (pCKII), formerly known as the plastid transcription kinase, was used as a first case study on this peptide microarray (Baginsky *et al.*, 1999). Once having a robust target set of pCKII substrates in hands the corresponding phosphorylation motif will be concluded. While pCKII is known to control plastid gene expression directly, indications for a long-term influence on plastid and nuclear gene regulation become apparent for the thylakoid associated kinase STN7 (Bonardi *et al.*, 2005; Bräutigam *et al.*, 2009; Pesaresi *et al.*, 2009). In order to unravel its direct or indirect activity on the plastid transcription system, comparative phosphoproteomic studies in *Arabidopsis thaliana* WT plants versus *stn7* [SALK_073254] T-DNA insertional mutants will be conducted. To this end a workflow was generated including an enrichment of the plastid genetic system by Heparin Sepharose®, followed by tryptic digestion, phosphopeptide enrichment and MS dissections. Due to complementing effects of the homologous STN8 kinase, the *stn8* [SALK_060869] and the *stn7stn8* double T-DNA insertional mutants will be included in these analyses. With the identified targets in hands phosphorylation motifs will be depicted.

The following chapter consists of four publications, which present my experimental progress in this field. The results are discussed and analyzed in chapter 3. Estimations on the influence of phosphorylation events in the plastid signal transduction system will be introduced.

2 publications

The following list summarizes my publications, which were generated on the chloroplast protein phosphorylation theme during my doctor of philosophy (PhD) time.

The first article reviews the state of the art knowledge on chloroplast phosphorylation networks at the beginning of my experimental work.

Schönberg, Anna; Baginsky, Sacha (2012): Signal integration by chloroplast phosphorylation networks: an update. *Frontiers in Plant Science*, 3, 256.

own contribution: establishment of the method

generation of the figures

development of the manuscript

The following two articles present the work on the peptide microarray ChloroPhos1.0. The first article focusses thereby on the experimental setup and wet lab method with this microarray. The PloS article presents the experiments and results of the pCKII-ChloroPhos1.0 project.

Schönberg, Anna; Baginsky, Sacha (2015): The peptide microarray ChloroPhos1. 0. A screening tool for the identification of Arabidopsis thaliana chloroplast protein kinase substrates. *Plant Phosphoproteomics: Methods and Protocols*, 147–157.

own contribution: establishment of the method

generation of the figures

development of the manuscript

Schönberg, Anna; Bergner, Elena; Helm, Stefan; Agne, Birgit; Dünschede, Beatrix; Schünemann, Danja; Schutkowski, Mike; Baginsky, Sacha (2014): The peptide microarray "ChloroPhos1.0" identifies new phosphorylation targets of plastid casein kinase II (pCKII) in *Arabidopsis thaliana*. *PloS One* 9 (10), e108344.

own contribution: establishment of the method

data analysis and interpretation

generation of the figures

development of the manuscript

The fourth publication presents the MS-based comparative phosphoproteomics approach for the identification of STN7 and STN8 targets.

Schönberg, Anna[1];Rödiger, Anja[1]; Mehwald, Wiebke; Galonska, Johann; Christ, Gideon; Helm, Stefan, Thieme, Domenika; Majovsky, Petra; Hoehenwarter, Wolfgang; Baginsky, Sacha (2017): Identification of STN7/STN8 kinase targets reveals connections between electron transport, metabolism and gene expression. *The Plant journa,l* 90 (6), 1176–1186.

own contribution: establishment of the wet lab method

              data analysis

              generation of the figures

              development of the manuscript

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[1] Both authors contributed equally to this work.

2.1 Signal integration by chloroplast phosphorylation networks: an update

2.1.1 Summary

The following review presents an overview of plastid protein phosphorylations in *Arabidopsis thaliana*, which innervate modifications or linkage of distinct signaling pathways. Therefor the well characterized protein kinases STN7, STN8, pCKII and CSK and the protein phosphatases PPH1 and PBCP are presented with their corresponding targets. Connections between the plastid metabolism and adaptations of the plastid and nuclear gene expression are delineated.

The reader is introduced into the field of chloroplast phosphorylation networks by the state transition kinase STN7 (Allen *et al.*, 1981; Bennett, 1977). Its activity on LHCII, which leads to the transition from state 1 to state 2 is described (Lemeille & Rochaix, 2010). Beside its influence on the short-term adaptation to prevailing light conditions is a further function on the long-term acclimation indicated, which results in the adjustment of the photosystem stoichiometry (Bräutigam *et al.*, 2009). Possibilities of STN7 induced signal transductions to the chloroplast and nuclear gene expression system are discussed. The thylakoid soluble phosphoprotein 9 (TSP9) and the transcriptionally active chromosome 16 protein (TAC16) are presented as further targets of this kinase, which broadens its substrate spectrum (Ingelsson & Vener, 2012).

STN8, a homolog of STN7, is presented in this review as the PSII core kinase, which is active under high light (Pesaresi *et al.*, 2011). CP43, D1, D2 and PsbH are listed as typical targets of STN8 (Bonardi *et al.*, 2005; Vainonen *et al.*, 2005; Vainonen *et al.*, 2008). Furthermore are Cas and PGRL1a presented as STN8 substrates. Cas is shown to control high light induced changes in gene expression and the phosphorylation of PGRL1A is presented as an inductor of faster switches from linear electron flow (LEF) to cyclic electron flow (CEF) (DalCorso *et al.*, 2008; Ingelsson & Vener, 2012; Petroutsos *et al.*, 2011; Reiland *et al.*, 2011). Beside this is a further influence of STN8 on the LTR mentioned, similar to that of STN7 (Bonardi *et al.*, 2005; Bräutigam *et al.*, 2009; Pesaresi *et al.*, 2009).

The stromal pCKII kinase is characterized as pleiotropic due to its diverse substrate spectrum (Kanekatsu *et al.*, 1998). It is supposed to connect chloroplast homeostasis with the regulation of transcription (Baginsky *et al.*, 1999). Many components of the plastid gene expression system are described as targets for pCKII, which becomes inactivated by reduced glutathione (Baginsky *et al.*, 1999). The idea of a regulatory unit consisting of CSK and pCKII, which controls plastid transcription, is described (Reiland *et al.*, 2009). CSK on its own is termed to resemble two component sensor

kinases from prokaryotic origin, which needs accordingly a redox regulator. Furthermore is CSK suspected to be the redox sensor of the Co-localization for Redox Regulation (CoRR) hypothesis (Puthiyaveetil *et al.*, 2012).

The complexity of the phosphorylation induced signaling becomes exemplified by the presentation of inter-kinase connections as network nodes. In this respect is the STN7 kinase described as a substrate of pCKII, which doesn´t affect its activity, but its stability (Willig *et al.*, 2011).

In order to enlarge the view on this network further kinases, like TAK1 operating on the thylakoid proteins and ABC1K located at the plastoglobuli, are noted (Lundquist *et al.*, 2012; Puthiyaveetil *et al.*, 2008; Vidi *et al.*, 2006). In addition is a calcium stimulus dependent phosphorylation depicted, whereat the responsive kinases are elusive so far (Nomura *et al.*, 2012; Sai & Johnson, 2002; Stael *et al.*, 2012).

Since protein phosphorylations are reversible PTMs, phosphatase activities need to be regarded for understanding the diversity of the signal integration. Two phosphatases are included in this literature review. On the one hand is PPH1/ TAP38 presented, which is located at the thylakoid membrane and dephosphorylates STN7- dependent LHC components (Pribil *et al.*, 2010; Shapiguzov *et al.*, 2010). On the other hand the PBCP is described. Basically this phosphatase is characterized by its counteracting activity of STN8 (Samol *et al.*, 2012; Shapiguzov *et al.*, 2010). According to the ATTEDII coexpression database are correlations between PBCP, STN8, CSK and sigma factor 3 (SIG3) noted as possible co-regulational connections.

Beside such clear-cut enzyme substrate relationships a functional overlap for some kinases and phosphatases is mentioned, e.g. for PPH1 and PBCP.

Beyond these enzyme substrate relationships also methods and experimental setups, which are available in the community, are presented in this article.

2.1.2 Article

This is a place holder for the following article:

Schönberg A., Baginsky S. (2012) Signal integration by chloroplast phosphorylation networks: an update. *Frontiers in plant science*, **3**, 256.

doi: 10.3389/fpls.2012.00256

2.2 The Peptide Microarray ChloroPhos1.0: a screening tool for the identification of *Arabidopsis thaliana* chloroplast Protein kinase substrates

2.2.1 Summary

Understanding the role of a kinase in a signal transduction network is impossible until some substrates are known. In order to unravel protein kinase substrate connections in the chloroplast signaling network the peptide microarray ChloroPhos1.0 got established as an in vitro screening tool. Based on all available *Arabidopsis thaliana* phosphoproteomics data, a peptide library consisting of chloroplast protein sequences was generated. By the status of January 2012 376 chloroplast phosphoproteins were known. For the generation of the peptide library 15mer sequences of these phosphoproteins with the phosphosite centered were taken including some variations. The resulting set of 905 peptides was expanded by microarray production and phosphorylation controls. Peptide synthesis and microarray production was outsourced to Steinbeis GmbH. Each peptide was spotted for control reasons in triplicate next to each other and the entire set was printed in 3 identical subarrays on one microarray.

Initial incubations with chloroplast protein extracts showed despite phosphorylation activity on extract own proteins none on the immobilized peptides. The latter were phosphorylated with preparations of higher specific kinase activity, which were yielded by affinity enrichment of native protein extracts and their recombinant overexpressed analogs.

The following article presents the setup and method of this peptide microarray for users. Detailed experimental and analysis instructions including handling hints are described. Thereby two options for the imaging process are presented: one is using Phosphorimaging after radioactive phosphate donor usage and the other one is using a fluorescence readout after incubation of the microarray with the Phosphoprotein stain Pro-Q^{TM} diamond post reaction. (Baginsky, 2016; Baginsky & Gruissem, 2001; Henkel *et al.*, 2016)

2.2.2   Article

This is a place holder for the following article:

Schönberg A., Baginsky S. (2015) The peptide microarray ChloroPhos1. 0: a screening tool for the identification of Arabidopsis thaliana chloroplast protein kinase substrates. *Plant Phosphoproteomics*. Humana Press, New York, NY, pp. 147-157.

doi: 10.1007/978-1-4939-2648-0_11

2.3 The Peptide Microarray "ChloroPhos1.0" Identifies New Phosphorylation Targets of Plastid Casein Kinase II (pCK II) in *Arabidopsis thaliana*

2.3.1 Summary

The former article introduced ChloroPhos1.0 as new tool and method for unraveling chloroplast protein kinase substrates. The plastid casein kinase II was chosen as a first case study for substrate identifications on this peptide microarray. The following article presents the construction of the peptide chip and the generation of native and recombinant pCKII preparations, which are incubated on it and analyzed for their activity.

The establishment of the peptide microarray ChloroPhos1.0 is described starting with the generation of the knowledge based peptide library. Rules for this library construction are presented in detail. The peptides were synthesized with the N-terminal linker N-(3-(2-(2-(3-amino-propoxy-)-ethoxy)-ethoxy)-propyl)-succinamic acid via spot technology on cellulose membranes, cleaved and printed on epoxy-functionalized glass slides (Frank, 1992). The chemistry of the peptide synthesis and a scheme of the array layout are depicted in the following article.

First kinase activity trials with incubations of chloroplast protein extracts on the microarray failed in terms of signal detection. Preparations of chloroplast kinases with higher specific activity were needed. Therefor the already well characterized pCKII was sighted as a first case study. Formerly pCKII was termed plastid transcription kinase due to its activity on the chloroplast gene expression system. In order to enlarge the knowledge on its substrates, and understand plastid signal integration between gene expression and e.g. photosynthesis, incorporation of pCKII on ChloroPhos1.0 was aimed. For this purpose the native pCKII was enriched using Heparin Sepharose® CL6B affinity chromatography on *Sinapis alba* and *Arabidopsis thaliana* chloroplast protein solubilisates. For control reasons a recombinant maltose-binding protein (MBP)-tagged pCKII version, which was generated by overexpression in *Escherichia coli,* was included in these studies. Those preparations were tested for kinase activity in batch mode using dephosphorylated, heat inactivated chloroplast proteins as substrates and were analyzed for their protein composition via a liquid chromatography-high definition-mass spectrometry[E] (LC-HD-MS[E]) approach. All three preparations phosphorylated a similar substrate set on the peptide chip, which was different to that of the control bovine heart protein kinase A (PKA) supporting the specificity of the pCKII preparations on the immobilized peptides. By the alignment of these pCKII target phosphorylation sites a motif with

acidic amino acid residues, e.g. aspartic and glutamic acid, in the +1 to +3 positions relative to the phosphorylation site appeared. The ChloroPhos1.0-pCKII substrate set encompasses targets of the plastid gene expression system, e.g. the translation elongation factor 1B (EF1B), the DEAD box RNA helicase 3 (RH3) or TAC10, a component of the RNA polymerase complex. In addition to such typical targets also substrates belonging to energy metabolism were found, e.g. albino 3 (Alb 3) which is essential for photosynthesis, more precisely the LHC integration into thylakoids. Alb 3 got further characterized by using recombinant phosphosite mutant proteins and performing kinetic studies on its WT protein version. Only in the pCKII activity assay with the phosphosite mutant serine 424 alanine (S424A) of Alb 3 the signal was completely lost, indicating serine 424 as the phosphosite of pCKII in this target.

The use of ChloroPhos1.0 as a screening tool for the identification of plastid kinase substrates is shown using the example of pCKII. Furthermore advantages and drawbacks of this system are discussed.

2.3.2 Article

This is a place holder for the following article:

Schönberg A., Bergner E., Helm S., Agne B., Dünschede B., Schünemann D., Schutkowski M., Baginsky S. (2014) The peptide microarray "ChloroPhos1.0" identifies new phosphorylation targets of plastid casein kinase II (pCKII) in Arabidopsis thaliana. *PloS one*, **9**, e108344.

doi: 10.1371/journal.pone.0108344

2.4 Identification of STN7/STN8 kinase targets reveals connections between electron transport, metabolism and gene expression

2.4.1 Summary

The former described analyses are based on in vitro experiments with pCKII preparations on the peptide microarray ChloroPhos1.0. All attempts to analyze the activity of the thylakoid membrane associated kinases STN7 and STN8 on this microarray failed so far due to insufficient kinase activities of the conducted protein preparations. In order to analyze their activity an in vivo approach, which is based on mass spectrometry detections, was pursued. The following study describes a special comparative phosphoproteomics setup, which enabled the identification of so far unknown STN7 and STN8 targets. STN7 is well known to be responsible for state transitions. In this process it phosphorylates LHCII components and enables a short term acclimation of the photosynthetic machinery to prevailing light conditions (Bellafiore *et al.*, 2005; Depège *et al.*, 2003; Rochaix, 2007; Tikkanen *et al.*, 2012). Beyond this interaction with thylakoid proteins an influence of this kinase on the so called long term response (LTR), which affects the nuclear and chloroplast gene expression, was shown (Pesaresi *et al.*, 2009). If this influence is a direct one by phosphorylation of gene expression components or if it is a more indirect connection due to changes in redox homeostasis is so far unknown (Bräutigam *et al.*, 2009; Pfannschmidt, 2010). STN7 and its paralog STN8 show a substrate overlap to some extent (Rochaix, 2014). STN8 is in difference to STN7 known to act on photosystem II core components, which influences thylakoid membrane folding and the PSII repair cycle (Bonardi *et al.*, 2005; Fristedt *et al.*, 2009; Tikkanen *et al.*, 2008; Vainonen *et al.*, 2008; Wunder *et al.*, 2013). Unraveling an influence on chloroplast gene expression components by STN7 and STN8 is aimed with the experimental design. The procedure consists in a first step of chloroplast isolations from WT and *stn7/ stn8* deficient *Arabidopsis thaliana* plants. After depletion of highly abundant photosynthetic proteins via Heparin Sepharose® affinity enrichment, the remaining plastid proteins were tryptic digested and enriched for their phosphopeptides by titanium dioxide (Baginsky *et al.*, 1999; Kelstrup *et al.*, 2013; Schönberg *et al.*, 2014). Electrospray ionization (ESI)-mass spectrometric analyses were executed using two fragmentation techniques, i.e. collision induced dissociation (CID) and multi stage activation (MSA); and two different analysis software tools, i.e. Sequest and MaxQuant.

The four phosphoproteins: ribonucleoprotein particle 29 (RNP29), RbcL, chloroplast ribosomal protein S7 (Rps7) and the ferredoxin-NADP$^+$ oxidoreductase (FNR) fit to the chosen stringent

selection criteria for STN7/STN8 substrates. A label free quantification is performed on them. Extracted ion chromatograms are searched for signal intensities corresponding to the phosphopeptide in the accurate mass bin. The mass bin for these four newly identified STN7/STN8 targets is empty in the TiO_2 enriched samples of the double mutant while signals are found in WT samples.

In order to unravel STN7 or STN8 responsibility on these four substrates a comparative phosphoproteomics analysis between WT and the *stn7* and *stn8* deficient single mutants was performed. Therefor chloroplast proteins were tryptic digested and enriched for phosphopetides by titanium dioxide-metal oxide affinity chromatography (TiO_2-MOAC) (Kelstrup *et al.*, 2013). These samples were further searched for the phosphopeptides via an accurate inclusion mass screening (AIMS) (Jaffe *et al.*, 2008). This shows that the phosphorylation of FNR and Rps7 is strictly dependent on STN7. The RbcL phosphopeptide was identified at low-abundance in one biological replicate of the *stn7* background, which indicates that STN8 is in charge at lower efficiency. In contrast the RNP29 phosphopeptide was detected in all three biological replicates of the three genotypes, indicating cross phosphorylation activity of STN7 and STN8.

Via complementary fragmentation data from MS/MS spectra of the chloroplast sample a set of LHC-phosphorylation sites was assembled for a phosphorylation motif assignment. These sequences contain a serine or threonine as phosphorylation residues with a glycine, proline or arginine in the −1 position. By aligning the new identified phosphopeptides a GS phosphorylation motif is found in three out of the five sequences. This motif is similar to that of LHCs indicating primarily a STN7 activity.

With this glycine-serine/ glycince-threonine (GS/GT) phosphorylation motif in hands targeted screens for further STN7 and STN8 substrates were conducted on data of a large scale experiment (Roitinger *et al.*, 2015). 20 phosphopeptides were found by this search. Four out of these 20 peptides were identified in the chloroplast extracts by accurate inclusion mass screening (Jaffe *et al.*, 2008). Thereof the RNA binding protein (At2g37220) was identified as a STN8 and caseinolytic protease proteolytic subunit 3 (ClpP3) as a clear STN7 target.

Based on the newly identified targets of STN7 and STN8, their influence on photosynthetic electron transport, chloroplast metabolism and gene expression is discussed in the following article.

2.4.2   Article

This is a place holder for the following article:

Schönberg A., Rödiger A., Mehwald W., Galonska J., Christ G., Helm S., Thieme D., Majovsky P., Hoehenwarter W., Baginsky S. (2017) Identification of STN7/STN8 kinase targets reveals connections between electron transport, metabolism and gene expression. *The Plant Journal*, **90**(6), 1176-1186.

doi: 10.1111/tpj.13536

3 Discussion

3.1 ChoroPhos1.0 as an in vitro screening tool

3.1.1 Peptide microarrays: scope and pitfalls

In order to interpret the in vivo situation via peptide microarray based experiments some constraints have to be considered. Real reactant ratios and system level effects, e.g. the existence of kinases, phosphatases and proteases in parallel are not represented in the experimental setup. While tiny off-target effects are accepted in the cellular system due to the reversibility of phosphorylation, they lead to an overestimation of substrate diversity identified by peptide chip analyses. All peptides are presented in equal concentrations on the microarray. Whereat in the cellular system are protein concentrations an important regulator of the signal fine tuning. The usage of such microarrays can lead to false positive conclusions, because localization effects or substrate competition are not represented by this method (Ubersax & Ferrell, 2007). Enzymatic reactants in subcellular compartments can be furthermore so highly concentrated, that already a low binding capacity is sufficient for a successful phosphorylation reaction (Masch, 2016). Such effects are not presented by this array, which may lead to false negative interpretations.

Kinase substrate interactions occur in vivo on the protein level, where conformational aspects, e.g. a special three-dimensional (3D) structure of the targets can be determinants of the reaction. Substrate recognitions in vivo are primary based on interactions between the primary target sequence and the catalytic cleft of the kinase. Furthermore play distal docking sites in the targets, which can be some hundred amino acids apart of the phosphorylation site, an important role for a successful enzymatic reaction (Ubersax & Ferrell, 2007). Phosphorylation is in this case allosterically regulated via a raise of the reactants concentration (Ubersax & Ferrell, 2007). Besides such targeting subunits may scaffolding proteins direct the catalytic subunit to the reaction locus (Hubbard & Cohen, 1993). By using the microarray with the 15mer peptide sequences such effects are not present, which may lead to false negative assignments.

3.1.2 pCKII on ChloroPhos1.0

The plastid casein kinase II (pCKII) is one of five casein kinase II (CKII) alpha subunits encoded in the *Arabidopsis thaliana* genome, which is imported in the chloroplast (Salinas *et al.*, 2006). During incubations of pCKII preparations on ChloroPhos1.0 is a high level of specificity reached due to the

basic peptide library, which consists only of in vivo substrates. In order to proof the target specificity of the used pCKII preparations on the peptide chip a control experiment with a commercially available bovine heart protein kinase A (PKA- Sigma-Aldrich) was conducted. PKA showed a totally different substrate set and phosphorylation motif, which proofs the high specificity of the pCKII preparations on ChloroPhos1.0.

Incubations with chloroplast extracts and protein solubilisates failed in terms of signal detection on the microarray. By analyzing these plastid protein preparations after array incubation via SDS-PAGE based autoradiography screens, lots of signals on extract own proteins were detected for chloroplast solubilisates and stromal lysates. This argues for the described reasons of high false negative rates by such peptide array approaches. Modifications of assay components, e.g. improving the protein concentration, addition of cofactors, adjustments of the radioactive / non-radioactive ATP ratio and the choice of mild nonionic detergents didn´t improve signal detection on ChloroPhos1.0.

By the incubation of Heparin Sepharose® enriched chloroplast solubilisates on the microarray first signals of a native preparation were detectable. These preparations showed sufficient phosphorylation activity on the peptides, which is due to the absence of preferential protein targets. Heparin as a highly sulfated glucosaminoglykan has a polyanionic structure analogous to nucleic acids, which allows the purification of proteins typical for DNA- or ribonucleic acid (RNA)- interaction, e.g. kinases. As shown before in *Sinapis alba* this method can be used to enrich the chloroplast gene expression system including the plastid casein kinase II in a native and active conformation (Baginsky *et al.*, 1999). The eluates of this affinity enriched *Arabidopsis thaliana* chloroplast solubilisates were able to utilize ATP and GTP as phosphate donors and got inhibited in their kinase activity by heparin, which reveals the typical CKII specificity. By comparing the peptide chip results of this native enriched pCKII fraction with those of a recombinant overexpressed *Arabidopsis thaliana* pCKII version a stringent data set for this kinase was extracted. Some further peptides were exclusively phosphorylated by the recombinant pCKII preparation due to its higher specific activity. The stringent pCKII target set is smaller than the predicted one of Reiland and colleagues, which is based on a phosphorylation motif query (Reiland *et al.*, 2009).

Beside the expected high number of false negatives also false positives are apparent in the data set. E.g. the peptide of the envelope membrane protein translocon at the outer envelope membrane protein 159 (TOC 159) shows strong signals, but is assumed to be a false positive detection due to its position in the acidic A domain of the native protein, which reaches the cytosol (Agne *et al.*, 2010). Another false positive in the substrate set is the soluble N-ethylmaleimide-sensitive factor adaptor protein 33 (SNAP33) peptide, which is an endosomal contaminant in the chloroplast reference

proteome list and got included in the knowledge based peptide library for ChloroPhos1.0 (El Kasmi *et al.*, 2013). It is very likely that these proteins are targets for other CKII alpha subunits in the nucleus or cytosol (Salinas *et al.*, 2006). This observation shows the importance of reactant co- localization within a cell for a successful enzymatic reaction. Determining such contaminations in protein reference lists is a commonly discussed problem (Gingras *et al.*, 2005). It is also possible that some proteins stick to the envelope membrane outside of the chloroplast and get co-isolated and detected via mass spectrometry with the chloroplast proteins (Wienkoop *et al.*, 2010). Due to their MS-detected low concentration the assumption of facing a low abundant protein is reliable at a first glance. Only by the combination of different experimental methods, which provide similar results, the reliability of such conclusions is improved. Beside such outtakes the pCKII substrate set comprises of proteins functioning in gene expression, energy metabolism and signal transduction.

3.1.3   pCKII targets on ChloroPhos1.0

3.1.3.1 pCKII targets of the plastid gene expression system

The majority of the detected targets belong to the plastid gene expression system, e.g. the transcriptionally active chromosome protein 10 (TAC 10), an essential component of the plastid RNA polymerase complex with a S1 RNA binding domain (Pfalz & Pfannschmidt, 2013). The identified phosphorylation site of TAC10 is positioned in the C-terminal region downstream the S1 domain. This region is known to interact with PEP associated proteins (PAPs), which promotes PEP complex formation and a proper chloroplast development (Chang *et al.*, 2017). The detected TAC10 phosphorylation might alter the interaction with PEP associated proteins in order to regulate plastid development. Former studies on RNA polymerase complex phosphorylation by pCKII show clearly a decrease of in vitro transcription (Baginsky *et al.*, 1999; Puthiyaveetil *et al.*, 2012), which indicates a similar effect on this member of the transcriptionally active chromosome. In non- photosynthetic organs, like in roots, a higher concentration of pCKII is detected, which might prevent gene transcription once it is not necessary (Baerenfaller *et al.*, 2011). This supports the view on protein phosphorylation as a common feature in activity regulation of transcription factors or protein complex assemblies (Karin & Hunter, 1995). The transcription initiation factor IIF (At4g12610) is a further target detected in these studies, which complies with such criteria.

RH3, the DEAD box RNA helicase, is another target of the plastid casein kinase II on ChloroPhos1.0. It functions in post transcriptional processes, e.g. in the splicing of group II introns and plays a role during plant growth and salt or cold stress responses (Asakura *et al.*, 2012; Gu *et al.*, 2014). The

detected phosphorylation site is positioned close to the N-terminus of the mature protein, which makes an influence on catalytic processes very unlikely. An influence on the rearrangement of RNA structures or degradation processes is in fact much more realistic (Banroques *et al.*, 2011; Cordin *et al.*, 2006). The 31kDa RNA binding protein (RNP31) is another already well known target of pCKII, which functions in the gene expression system. RNP31 consists of an acidic domain, where the phosphorylation site is located, and two RNA recognition motifs (Kanekatsu *et al.*, 1993). It is important for the chloroplast resistance against cold stress by enabling mRNA editing and transcript stability due to interaction with RNA sequences at multiple sites (Kupsch *et al.*, 2012; Tillich *et al.*, 2009). Phosphorylation alters these RNA protein binding properties (Loza-Tavera *et al.*, 2006).

Beyond such targets involved in gene transcription the peptide of the EF1B showed strong signals on all peptide microarrays incubated with pCKII preparations. EF1B is as a component of the plastid 30S ribosome involved in ribosome biogenesis and it´s phosphorylation regulates protein-protein-interactions during the translational process (Horiguchi *et al.*, 2003).

3.1.3.2 pCKII targets involved in signal transduction

Beside proteins involved in gene expression, two substrates were detected functioning in cellular signal transduction. Thereof one is the mechanosensitive ion channel protein 3 (MSCS-3) (At1g58200), which is located mainly in the plastid envelope membrane. In response to tension changes in the envelope membrane it forms a channel for the ion release out of plastids, which protects plastids from hypoosmotic stress (Haswell & Meyerowitz, 2006; Jaffe *et al.*, 2008; Veley *et al.*, 2012). Furthermore it relays a mechanical signal for the proper regulation of the filamenting temperature-sensitive mutant Z (FtsZ) ring formation during chloroplast divison under normal plant growth (Wilson *et al.*, 2011). Phosphorylation might alter its conformation and interactions with protein partners during signaling processes. The same holds true for the chloroplast heat shock protein 70-2 (cpHsc70-2), a stromal heat shock protein, that is essential for a proper plant development and thermotolerance of germinating seeds (Su & Li, 2008). It acts as a chaperone in protein folding and transmembrane transport (Latijnhouwers *et al.*, 2010). Alterations of its enzymatic performance due to the described phosphorylation is very likely.

3.1.3.3 pCKII targets involved in energy metabolism

Plastid casein kinase II targets on ChloroPhos1.0 belonging to energy metabolism are the RbcS and RuBisCo activase, which act in carbohydrate fixation; and Alb 3, which is important in photosynthesis. The small subunit of RuBisCo (At5g38410) is necessary for the RuBisCo assembly, where

phosphorylation is supposed to have an influence (Izumi *et al.*, 2012). RuBisCo activase removes inhibitory phosphate esters from the active site of RuBisCo and coordinates by this reaction the active state of this enzyme and the rate of electron transport (Kim *et al.*, 2016). The phosphorylation is found in many phosphoproteomics studies in *Arabidopsis thaliana* and occurs especially by a light to dark shift (Agrawal *et al.*, 2012; Meyer *et al.*, 2012; Reiland *et al.*, 2009; Reiland *et al.*, 2011; Wang *et al.*, 2014). The phosphorylation site Thr-78 lays in the N-terminal domain of the protein, which is known to be essential for its interaction with RuBisCo (Allen *et al.*, 1981; Salvucci & Klein, 1994; Stotz *et al.*, 2011). Kim and colleagues showed in their study that this pCKII phosphorylation is not essential for the RuBisCo activity control and postulated a regulatory role during the absence of redox control or under stress conditions (Kim *et al.*, 2016).

Analyses on ChloroPhos1.0 unraveled Alb 3 as a so far unknown substrate of pCKII. It belongs to the membrane protein insertase family and consists of 5 transmembrane domains, which form a positively charged hydrophilic cavity and enable the integration of light harvesting complexes (LHCs) into the thylakoid membrane (Kumazaki *et al.*, 2014; Moore *et al.*, 2000; Woolhead *et al.*, 2001). This aligns with the fact that a lack of Alb 3 results in an albino phenotype (Sundberg *et al.*, 1997). Further investigations identified Alb 3 as an auxiliary protein for the photosystem II assembly (Schneider *et al.*, 2014). The detected phosphopeptides for Alb 3 on the peptide chip consist of three possible serine phosphorylation sites. In order to determine the exact phosphorylation position of pCKII an in vitro kinase activity assay with the recombinant overexpressed and affinity purified Alb 3 protein was performed. In parallel the same reaction was conducted with phosphosite mutant proteins, where the phosphorylatable serines are exchanged to alanine as single or triple mutant versions. Ser 424 got established as the major phosphorylation site by these investigations. This phosphosite is located at the C-terminal stroma exposed region, which maybe affects the interaction with the LHCs during their integration into thylakoids. Multiple sequence alignments using *Arabidopsis thaliana* and evolutionary relatives show a conservation of this phosphosite in *Zea mays*, *Hordeum vulgare* and one *Oryza sativa* homolog, but in *Pisum sativum*, *Solanum tuberosum* and *Chlamydomonas reinhardtii* this phosphosite is not existing due to different sequences. This indicates a specialization in regulation of LHC complex insertion into thylakoids.

The identified substrate diversity demonstrates the pleiotropic nature of pCKII, functioning in different plastid biochemical pathways and supporting a crosstalk between different chloroplast functions.

3.1.4 Identification of pCKII phosphorylation motifs by the use of ChloroPhos1.0 results

Utilizing kinase activity assays on microarrays narrows huge prediction target spectra down to a more realistic substrate set (Baginsky, 2016). With such a target set for a certain kinase in hands becomes phosphorylation motif analysis facilitated (Baginsky, 2009). Substrate recognition motifs for kinases span usually 4 amino acids up- and downstream to the phosphorylation site (Ubersax & Ferrell, 2007). The peptides on the ChloroPhos1.0 microarray exceed the length of typical docking sequences while spanning 7 amino acids up- and downstream to the phosphorylatable amino acid. Physical contact between protein kinase and peptides is possible due to complementary sequences in aspects of charge, hydrogen bonds and hydrophobic interactions. The plastid casein kinase II belongs to the class of serine threonine kinases and has in comparison to tyrosine kinases a relatively small catalytic cleft, which excludes tyrosine residues of its phosphorylation activity (Brown *et al.*, 1999; Hubbard, 1997). This view gets supported by the fact that no tyrosine phosphorylation was found in these analyses. Members of the CKII family are known to interact specifically with their subdomains VIII, I, II and III on acidic target sequences (Hanks *et al.*, 1988; Songyang *et al.*, 1996). In order to extract the phosphorylation motif of the used pCKII preparations the phosphorylated peptide sequences containing the phosphosites in their center got aligned. The WebLogo algorithm was used to visualize a motif sequence above the background, see **Figure 5** (Crooks *et al.*, 2004).

Figure 5 WebLogo of the pCKII substrates : Depicted is the weblogo for the consensus pCKII substrate set with centered phosphorylation sites of all three preparation types, which where incubated on ChloroPhos 1.0: i.e. the recombinant and native pCKII preparations of *Arabidopsis thaliana*, and the native one of *Sinapis alba* (Crooks et al. 2004). This graph is based on the one letter code of the amino acids and is illustrated in 3 bits latitude. Phosphorylatable amino acids are depicted in green, acidic ones in red, alkaline ones in blue and all other hydrophobic or neutral amino acids are shown as black letters.

In these sequence logos indicates the height of the symbols the relative frequency of each amino acid at the positions. The native and recombinant *Arabidopsis thaliana* pCKII preparations show a clear preference for acidic residues, e.g. aspartate and glutamate, proximal to the phosphosite. Thereby such acidic residues span in the WebLogo motif from -2 N-terminal to +3 C-terminal of the

$_{phospho}$serines. This aligns with former published CKII motifs containing aspartate and glutamate in positions neighbouring the $_{phospho}$serine (Kreegipuu *et al.*, 1998; Schutkowski *et al.*, 2004).

3.2 Mass spectrometry based Identification of new STN7 and STN8 kinase targets

3.2.1 Comparative phosphoproteomics: scope and pitfalls

The substrate spectrum of kinases and phosphatases can be detected by mass spectrometry based comparative phosphoproteomics. Comparative analyses are based on the comparison of the situation in WT versus mutant samples (Rochaix, 2014). Such comparisons are reliable once the qualitative status of the organisms with different genotypic backgrounds is similar under the chosen growth or treatment conditions in terms of physiological, metabolic and biochemical aspects. Comparative phosphoproteomics rely on the assumption that the phosphorylation rate of kinase targets in vivo is lower in kinase mutant samples than in the WT ones. The possibility that further enzymes, acting downstream on the kinase of interest, cause the phosphorylation rate of the identified substrates cannot be depicted via such approaches. **Figure 6** presents a scheme of complex protein interactions, which might contribute to the substrate phosphorylation level under a specific situation.
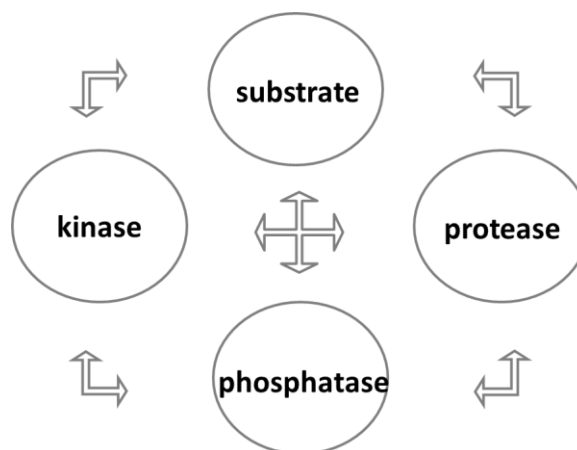
Figure 6 System level effects : Depicted are proteins of varying functions and their interaction possibilities. This graphic underlines the high complexity of enzymatic reaction networks, which cause a certain phosphorylation state in vivo.

3.2.2 Development of an experimental procedure for unraveling so far unknown STN7 and STN8 substrates

3.2.2.1 Plant material and growth conditions

The chosen soil growth and white light short day conditions allow the analysis of physiological relevant phosphorylation events, which is a prerequisite for this type of studies (Chitteti & Peng, 2007). The *stn7/stn8* double mutant was used for initial screening in these experiments, because STN7 and STN8 can complement each other to a certain extent (Bonardi *et al.*, 2005; Samol *et al.*, 2012; Vainonen *et al.*, 2008). Conclusions on the kinase substrate interactions can only be drawn once the kinases are active under the chosen growth conditions. STN8 is known to be active under a broad variety of prevailing light conditions, even under high light (Pfannschmidt & Yang, 2012; Puthiyaveetil *et al.*, 2012; Rochaix, 2011). In contrast is the STN7 activity restricted to low light conditions when the plastoquinone pool is partially reduced (Rintamaki *et al.*, 1997; Shapiguzov *et al.*, 2016; Tikkanen *et al.*, 2008). In order to proof STN7 activity a BN-PAGE with digitonin solubilized chloroplast extracts of all plant genotypes was conducted. Thereby the PSI-LHCI-LHCII supercomplex was visible in WT and *stn8* single mutants, but not in STN7 absent genotypes. This supercomplex exists only under state 2, which is caused by STN7 activity and starts with their phosphorylation of the light harvesting complex II protein 2 (Lhcb2) protein (Longoni *et al.*, 2015; Pesaresi *et al.*, 2009). As a second proof of the STN7 activity was a $_{phospho}$Lhcb2 Western Blot analysis conducted and revealed again STN7 activity in WT and *stn8* mutants.

3.2.2.2 Shrinking of sample complexity for a tailored phosphoproteomics analysis

One of the basic limitations in mass spectrometric phosphoproteomics is the high dynamic range of protein concentrations within a cell or their subcompartments (Baginsky & Gruissem, 2006; Jünger & Aebersold, 2014; Tichy *et al.*, 2011). Thus fractionation of proteins or peptides prior to MS analysis improves the detection of low abundant targets and increases the proteome coverage (Baginsky & Gruissem, 2006; Pinkse & Heck, 2006). A specific workflow for the identification of a STN7/STN8 influence on the chloroplast gene expression system was established in this study. This experimental setup consists of chloroplast isolations in a first step followed by a Heparin Sepharose® enrichment, which depletes highly abundant photosynthetic proteins (Schönberg *et al.*, 2014; Schönberg & Baginsky, 2012). The high concentration of transcriptional and translational components in these Heparin Sepharose® eluates approve the effect of the enrichment strategy. The eluate proteins were tryptic digested and enriched for their phosphopeptides by titanium dioxide (TiO_2) (Beltran & Cutillas,

2012; Kelstrup *et al.*, 2013; Larsen *et al.*, 2005). TiO_2 is used to adsorb phosphopeptides through a bidentate interaction (Kweon & Håkansson, 2006; Larsen *et al.*, 2005). Phosphopeptide enrichments prior to electrospray ionization –mass spectrometry (ESI-MS) are necessary, because phosphorylations occur in the most cases only substochiometric, which impedes their detection. These enrichment steps were successful, since more phosphopeptides were detected in TiO_2 enriched Heparin Sepharose® eluates than in TiO_2 enriched chloroplast extracts. The reduced sample complexity resulted in a higher dynamic range and allowed the identification of targets which usually escape detection.

3.2.2.3 Improvement of detections by a variation of MS fragmentation techniques

Two different fragmentation techniques were used in these ESI-MS based identifications. One is the collision induced dissociation (CID) and the other one the multistage activation (MSA). In the CID mode the protonated peptides are accelerated by electric potential in vacuum and collide with inert neutral gas molecules (Couto *et al.*, 2018). The kinetic energy is then converted into internal energy which is distributed over the whole molecule and causes the peptide ion to fragment at amide bonds along the backbone, which results in b- and y-ion series (Wells & McLuckey, 2005). $_{Phospho}$serine and $_{phospho}$threonine- containing peptides show in the positive ion mode the predominant loss of 98Da which corresponds to the neutral loss of phosphoric acid due to ß-elimination (DeGnore & Qin, 1998; Mann *et al.*, 2002; Tholey *et al.*, 1999). In order to deepen the phosphosite detection were MSA fragmentations performed. Thereby were the neutral loss produced ions activated and fragmented, which leads to further peptide backbone cleavages (Wells & McLuckey, 2005; Wu *et al.*, 2013). Two analysis software tools were used, i.e. Proteome Discoverer with the Sequest algorithm and MaxQuant (Cox & Mann, 2008). Phosphopeptides were assessed as reliable once they were detected with both scan types and analysis tools under stringent filter criteria, i.e. a low FDR of 1%, small precursor mass tolerances of 10 ppm and fragment ion tolerances of 0.8 Da. Thus the result is a very stringent data set.

3.2.2.4 Quality checks on kinase target assignments

Several controls on the experimental pipeline were conducted for the verification of substrate annotations. Phophopeptide detections were only classified as true, when they were detected in experiments with different MS-fragmentation techniques and via both software tools for data analysis. This rule shrinked the number of false positives. Further it was necessary to exclude any inclinations of the affinity enrichment steps in different genotypic backgrounds. Therefor the search

for the non-phosphorylated counterparts of the phosphopeptides in tryptic digested Heparin Sepharose® eluates was aimed. For the four substrates: RNP29, FNR, RbcL and Rps7 similar detection levels in WT and the stn7/ stn8 double mutant context were recorded. This underlines the fact that their lack of detection after the enrichment is due to missing phosphorylations in the double mutant samples. Beside this was a label-free quantification by HD-MS^E performed on these four substrates as a further control step (Helm *et al.*, 2014). Based on the extracted ion chromatograms the signal intensities of the phosphopeptides were searched in the accurate mass bin of the tryptic digested Heparin Sepharose® eluates in the WT and the double mutant context. The RCA phosphopeptide, which is known to be a clear pCKII target, was included in these searches as a negative control for the STN7 or STN8 kinase activity. The Lhcb4 phosphopeptide as typical STN7 target was added as a positive control. By setting a tolerance window of 3 Dalton (Da) and including the entire isotopic envelope the mass bin for the newly identified substrates and Lhcb4 was empty in the double mutant. On the contrary were comparable counts for the RCA phosphopeptide detected in both genotypes. This HD-MS^E mode reaches a high sensitivity and reliability once three technical replicas are analyzed (Helm *et al.*, 2014). This excludes any possible experimental bias based on the TiO$_2$ enrichment. In a further step the phosphopeptide occurrence in the chloroplast extracts independent of all enrichment steps was questioned. Therefor both software tools were used. Due to a masking of low abundant proteins by the high abundant photosynthetic ones a search for the targets was rendered in the WT and double mutant chloroplasts. The mass tags for all new identified substrates were found in WT chloroplasts but not in the ones of the double mutant. This strongly hints on STN7 or STN8 phosphorylation activity on these substrates. In order to distinguish between STN7 and STN8 activity an accurate inclusion mass screening (AIMS) was performed on TiO$_2$ enriched chloroplast proteins of WT, *stn7* and *stn8* single mutants (Jaffe *et al.*, 2008). Thereby masses of an inclusion list are monitored on each scan and MS/MS spectra are only acquired once an entry of the list is detected by its accurate mass and charge state (Jaffe *et al.*, 2008). This type of targeted analysis shows a high degree of sensitivity and allowed a clear statement for the kinase-substrate interactions.

3.2.2.5 STN7 and STN8 phosphorylation motif analysis and targeted screening for further substrates

For the determination of a STN7 and STN8 phosphorylation motif a set of phosphorylation sites for thylakoid associated proteins was collected (Reiland *et al.*, 2011; Roitinger *et al.*, 2015; Ubersax & Ferrell, 2007). Therefor complementary fragmentation data from MS/MS spectra of chloroplast samples were utilized. Peptide sequences containing a glycine, proline or arginine prior to a

$_{phospho}$serine or $_{phospho}$threonine predominated this collection. Three of the five new substrates also show a glycine at the -1 position followed by a $_{phospho}$serine. The similarity to the phosphorylation motif for the LHCIIs lead to the conclusion that the LHCII kinase: STN7 is responsible for these phosphorylations. In order to enlarge the substrate set of the STN7 and STN8 kinase a targeted search for this GS/GT motif was conducted. Therefor an independent large-scale *Arabidopsis thaliana* phosphoproteomics dataset was screened for this phosphorylation motif. This quantitative phosphoproteomics study was conducted for decoding the regulation of the ataxia telangiectasia-mutated (ATM) and the ataxia telangiectasia mutated and rad3-related (ATR) dependent DNA damage response in *Arabidopsis thaliana* (Roitinger *et al.*, 2015). The dataset comprises of 15445 unique phosphopeptides and can serve as a useful resource for *Arabidopsis thaliana* phosphosites. The authors reached this high level of detections by using a combination of phosphopeptide enrichments, i.e. strong cation exchange chromatography (SCX), IMAC and MOAC and the isobaric tags for relative and absolute quantitation (iTRAQ) method for peptide quantification (Agrawal *et al.*, 2012; Leitner, 2010; Villén & Gygi, 2008; Wiese *et al.*, 2007; Wolschin *et al.*, 2005). Twenty phosphopeptides containing the GS/GT motif were identified by this search in that large-scale dataset. These 20 peptides were retrieved in the chloroplast extracts of the WT plants, aswell as the *stn7* and *stn8* single mutants via an accurate inclusion mass screening (AIMS) (Jaffe *et al.*, 2008). Four out of 20 peptides were identified in the chloroplast extracts, thereof one was detected as STN7 and another one as a clear STN8 target. Phosphorylation motifs as fingerprints of their kinases, can be used to enlarge target searches, which is successfully shown in this study (Amanchy *et al.*, 2007; Schwartz & Gygi, 2005; Ubersax & Ferrell, 2007). Such targeted analyses facilitate the detection of low abundant peptides and raise thereby the dynamic range of the MS detection (Picotti *et al.*, 2009). The combination of different methods and strategies allows a deep view into the present phosphoproteomes.

3.2.3 STN7 and STN8 substrates functioning in diverse signaling pathways

3.2.3.1 STN7 and STN8 targets involved in energy metabolism

In the conducted AIMS analysis with the single mutants the ferredoxin dependent NADP reductase (FNR) was identified as clear STN7 target. FNR is a soluble protein, which becomes recruited to the thylakoid membrane via the translocon at the inner chloroplast envelope membrane protein 62 (TIC 62) and thylakoid rhodanese like (TROL) proteins (Mulo, 2011). TIC62 is a chaperone for FNR and protects it from degradation or inactivation during inactive photosynthesis (Benz *et al.*, 2009). FNR is the terminal enzyme of the photosynthesis under linear electron flow, where its C-terminus interacts

with TROL and supports the oxidation of ferredoxin and the reduction of $NADP^+$ to NADPH (Jurić *et al.*, 2009). NADPH is necessary for the carbon assimilation in the Calvin Benson cycle (Batie & Kamin, 1984; Dorowski *et al.*, 2001; Hermoso *et al.*, 2002). The detected phosphosite is distinct of the NADPH binding site. In vitro phosphorylation assays with FNR show that the phosphorylation modifies the ferredoxin FNR interaction and changes the PSI activity (Hodges & Miginiac-Maslow, 1993). Thus FNR phosphorylation is a regulatory network node between the photosynthetic electron flow and the demand for NADPH. FNR abundance and activity is in general under strong regulation in terms of gene expression, protein import, subchloroplastic localization and post translational modification (Benz *et al.*, 2009; Gummadova *et al.*, 2007; Hanke *et al.*, 2005; Kikuchi *et al.*, 2006; Lehtimaki *et al.*, 2014).

Another STN7 and STN8 target detected in this comparative MS-analysis is the large subunit of RuBisCo (RbcL). RuBisCo is the main enzyme of the Calvin Benson cycle, which supports its initiation by the addition of one molecule carbon dioxide to one molecule ribulose-1.5-bisphosphate. This stromal substrate must come into contact with the thylakoids, where it can serve as a substrate for STN7 and STN8. This interaction might occur during protein synthesis at polyribosomes, which are located at the thylakoid membrane (Zhang *et al.*, 2000; Zoschke & Barkan, 2015). Such polyribosomes encompass not only transcripts of membrane proteins but also the messenger RNA (mRNA) of RbcL (Hattori & Margulies, 1986; Mühlbauer & Eichacker, 1999). Thereby becomes translation connected with the photosynthetic electron transport (Allahverdiyeva *et al.*, 2015; Zhang *et al.*, 2000; Zoschke & Barkan, 2015). The identified RbcL phosphorylation occurs only in substoichiometric amounts. If this low abundant post translational modification has an effect on carbon fixation remains still unknown. Differences in the carbohydrate metabolism are observed for the *stn7* mutants in comparison to the WT plants. These mutants showed no increased starch content during a PSI to PSII light shift in comparison to their WT samples (Bräutigam *et al.*, 2009). RuBisCo subunits are the targets of many kinases, like pCKII, STN7 and STN8, which shows the close connection between signal transduction processes and the Calvin Benson cycle (Schönberg *et al.*, 2014).

STN8 as the PSII core component kinase phosphorylates mainly D1 (Bonardi *et al.*, 2005; Vainonen *et al.*, 2008). Once D1 is phosphorylated the PSII repair cycle starts and the photodamaged PSII moves from grana to stroma lamellae, where it becomes disassembled and D1 gets degraded by FtsH and Deg proteases (Kato & Sakamoto, 2014; Tikkanen *et al.*, 2008; Wunder *et al.*, 2013). A newly synthesized D1 interacts with D2 and CP43 via disulfide bonds and is inserted into the truncated PSII

complex, which relocates thereafter to the grana thylakoids (Kato & Sakamoto, 2014; Puthiyaveetil *et al.*, 2013).

The in this study identified substrates: FNR and RbcL expand the knowledge on typical membrane bound proteins for STN7 and STN8.

3.2.3.2 STN7 and STN8 targets of the plastid gene expression system

Beside such typical STN7 and STN8 targets functioning in the light-dependent reaction or Calvin-Benson cycle an influence in the so called long term response (LTR) is reported for both kinases (Bonardi *et al.*, 2005; Bräutigam *et al.*, 2009; Pesaresi *et al.*, 2009). This LTR is triggered by a reduced plastoquinone pool and connects their kinase activity with plastid and nuclear gene expression. If STN7 and STN8 are directly phosphorylating components of the chloroplast gene expression machinery or if their LTR influence is a more indirect one by the alteration of the redox homeostasis or metabolism was questioned in this study (Bräutigam *et al.*, 2009; Pfannschmidt, 2010). No comparative phosphoproteomics analysis so far identified STN7 targets belonging to the transcriptional core of the plastid RNA polymerase complex (PEP), due to their low abundance beside high abundant photosynthetic proteins (Pfalz & Pfannschmidt, 2013). Performing a Heparin Sepharose® chromatography on the chloroplast solubilisates enabled the concentration of plastid gene expression system components, which eased their identification after a TiO_2 phosphopeptide enrichment.

This strategy in combination with the AIMS application lead to the identification of Rps7 as clear STN7 substrate. This stromal target might come into contact with STN7 by its translation at thylakoid bound polyribosomes (Zhang *et al.*, 2000; Zoschke & Barkan, 2015). Rps7 initiates ribosomal assembly in bacteria and regulates its own gene expression by binding its own mRNA (Wimberly *et al.*, 2000). Furthermore interacts Rps7 with large 3´domains of the 16S ribosomal ribonucleic acid (rRNA) (Wimberly *et al.*, 2000). The identified phosphosite at serine 93 is close to this rRNA contact site, which maybe influences its rRNA connection, resulting in altered ribosome assembly kinetics.

A further plastid gene expression target identified in this study is the ribonucleoprotein 29 (RNP29). Chloroplast RNPs are in general suspected to protect mRNA endonuclease sensitive sites via competitive binding processes (Teubner *et al.*, 2017). RNP29 is known for binding large overlapping sequences of mRNA under chilling conditions (Kupsch *et al.*, 2012). In such situations produces the PEP complex lower amounts of mRNA, whereas genes dependent on nuclear-encoded plastid RNA-polymerase (NEP) become overexpressed (Hajdukiewicz *et al.*, 1997). This leads to an imbalance of

the plastid proteome. The mRNA processing raections, like splicing or editing, are reduced under low temperatures, while degradation processes are enhanced. Transcript stability and integrity is guaranteed under such chilling conditions via the creation of mRNA-RNP29 conglomerates. RNP29 is a target of both kinases: STN7 and STN8. This supports a constant phosphorylation level of this substrate, even under conditions when STN7 is inactive, e.g. under high light.

This study further revealed a closely related homolog of RNP29 as a substrate for STN8, namely the RNA binding protein (At2g37720). For such phosphorylations is a close contact between thylakoid membranes and the nucleoid necessary. The PEP complex becomes anchored to the thylakoid membrane by TAC16 during chloroplast biogenesis (Ingelsson & Vener, 2012). These anchoring allows the thylakoid kinases to phosphorylate such targets of the stromal gene expression machinery.

3.2.3.3 STN7 and STN8 targets involved in proteolysis

In addition to the described targets two proteases were detected as substrates of the thylakoid kinases. The phosphopeptide of the FtsH11 was found in WT samples and is missing in the double mutant ones. In order to control the reliability of the experimental setup, the tryptic digested Heparin Sepharose® eluates were searched for the corresponding protein abundances via MS^E (Helm *et al.*, 2014). In case of FtsH11 these analyses failed in terms of detection. But the following AIMS study with the single mutants versus WT samples of the TiO_2 enriched Heparin Sepharose® eluates identified the FtsH11 phosphopeptide accurate as STN7 and STN8 target. In vitro studies on this protease revealed a chaperonine function (Di Zhang *et al.*, 2010). FtsH11 forms complexes in the envelope membrane and performs housekeeping proteolysis within this membrane, independent of other thylakoid associated FtsHs (Wagner *et al.*, 2012). It degrades damaged or misfolded proteins in the amphiphilic lipid bilayer and maintains thereby the integrity of envelope membrane protein complexes. Another protease identified in this screen is ClpP3 as clear STN7 target. The CLP protease family is located in the stroma and acts in diverse and pleiotropic pathways (Kim *et al.*, 2013). *ClpP3* mutants show a variety of pleiotropic phenotypes, which hinders direct conclusions on its target spectrum (Kim *et al.*, 2013). But a delayed leave development and flowering is consistent for many of them. ClpP3 is involved in the built up of the photosynthetic machinery during embryogenesis and allows a fast degradation of damaged or misfolded components in the thylakoid membrane. A loss of the ClpP3 protease capacity specifically affected a subset of chloroplast proteases, which hints on a controlled proteolysis network. If the D1 protein is a direct target of ClpP3 is still debated.

3.2.4 Phosphorylation motifs of the identified substrates

The presented phosphorylation motifs for the STN7 and STN8 dependent substrates in **Figure 7** are generated with the WebLogo algorithm based on the newly identified substrates (Crooks *et al.*, 2004).
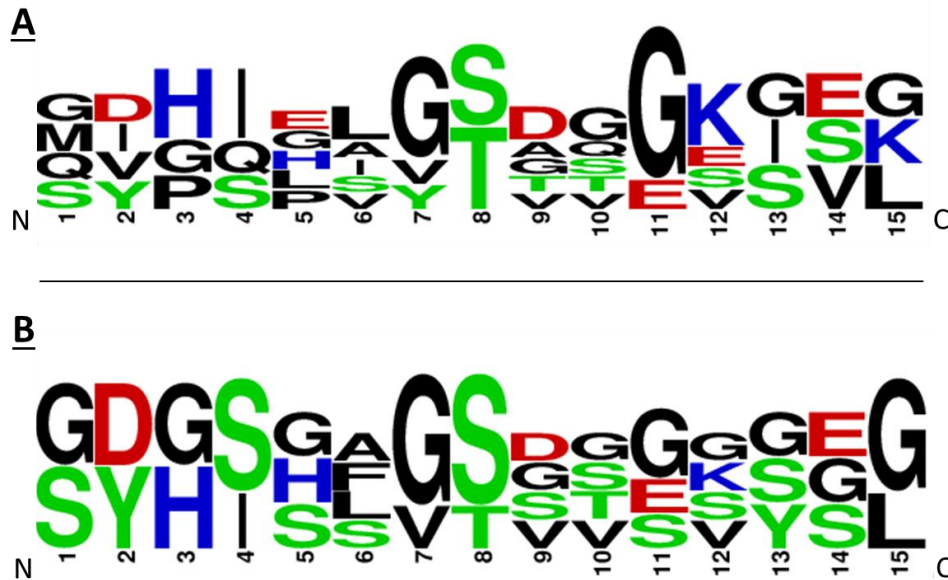
Figure 7 WebLogos of the STN7 and STN8 substrates : Depicted is the weblogo for the STN7-(A) and STN8-(B) dependent substrate set with centered phosphorylation sites in 15 mer sequences, which is identified by the phosphoproteomics pipeline of Heparin-Sepharose enriched chloroplast protein preparations (Schönberg et al. 2017, Crooks et al. 2004). All phosphopeptides, which were detected in the WT samples, but not in the stn7 (A) or stn8 (B) deficient mutants are used for the motif query. The graph is based on the one letter code of the amino acids and is illustrated in 4 bits latitude. Phosphorylatable amino acids are depicted in green, acidic ones in red, alkaline ones in blue and all other hydrophobic or neutral amino acids are shown as black letters.

These WebLogos are graphical representations of phosphorylation motifs within a multiple sequence alignment (Crooks *et al.*, 2004). Thereby indicates the height of each letter stack the sequence conservation at the certain position, whereas the height of each symbol within the stack reflects the relative frequency of the corresponding amino acid (Crooks *et al.*, 2004). Due to the substrate spectrum overlap of these stringent analysis data sets share the two phosphorylation motifs share basic characteristics. Glycine rich regions surrounding the phosphosite become apparent as favored phosphorylation sequences. Thereby such glycines span in the WebLogo motif from -1 N-terminal to +3 C-terminal of the $_{phospho}$serines or $_{phospho}$threonines. An aspartic acid in +1C-terminal and valines from -1N-terminal to +2 C-terminal of the phosphosite are further common features in this motif context. STN8 shows a stronger tendency to phosphorylate serine residues compared to STN7 based on this substrate set. The preference for glycine rich sequences in the STN7 context aligns with the

former identified LHC components, as earlier described (Cox & Mann, 2008; Ingelsson & Vener, 2012; Mann *et al.*, 2002; Reiland *et al.*, 2009; Reiland *et al.*, 2011; Reinl, 2008). For the former identified STN8 substrates Cas and CP43, the phosphomotif shows similar characteristics in respect of glycines at the -1 N-terminal position of the phosphorylation site (Meyer *et al.*, 2017; Reiland *et al.*, 2009). Since only a small number of substrates is identified with this stringent data set and within this more STN7 substrates are found, conclusions are only preliminary so far.

4   Summary of the results and outlook

All identified substrates for the three analyzed kinases of this PhD-thesis project are listed in the appendix. This table includes information on the experimental method and the possible function of the phosphorylation or the substrate. Analyses with the peptide microarray ChloroPhos1.0 revealed new insights into the substrate spectrum of the stromal pCKII, so far known as the plastid transcription kinase. In addition to components of the chloroplast genetic system, e.g. TAC10, RNP31, Ef1B and RH3, also substrates belonging to signal transduction were found, e.g. the MSCS like 3 and the heat shock protein cpHsc70-2. Furthermore targets were detected, which act in the energy metabolism were detected, like RCA, the SSU of RuBisCo and Alb 3. Alb 3 is necessary for the LHC integration into thylakoid membranes. The chloroplast phosphoproteomics study on the thylakoid associated kinases STN7 and STN8 revealed novel insights into their target spectrum. Thereby FNR and RbcL, which act in energy metabolism were found as targets for the state transition kinase STN7. Beyond this, also substrates belonging to the gene expression system were found for STN7, e.g. Rps7 and RNP29. Furthermore an activity of STN7 on the protease ClpP3 was detected. RbcL and RNP29 are dual specific targets, because the PSII core kinase STN8 can phosphorylate them in the absence of STN7. These analyses detected furthermore the RNA binding protein (At2g37220) as distinct STN8 substrate. Both studies enlarge the view on the activity of the three kinases: pCKII, STN7 and STN8 in the chloroplast signaling network.

With the peptide microarray ChloroPhos1.0 in hands further chloroplast kinase activities await now a detailed characterization. Active enzyme preparations of the atypical activity of BC1 complex (ABC1) kinases, thylakoid associated kinases (TAKs) and the two newly identified plastid localized protein kinases (At5g61560, At3g51990) are of interest for future peptide chip studies (Lundquist *et al.*, 2012; Lundquist *et al.*, 2013; Richter *et al.*, 2016; Snyders & Kohorn, 2001). Priming-, auto- or dephosphorylation events by protein phosphatases can be detected via different enzyme preparations in a row on this peptide microarray (Schutkowski *et al.*, 2004). Priming phosphorylations are often necessary to so called conditional docking sites, which turn into real docking sites once they carry a certain PTM, e.g. a phosphorylation (Ubersax & Ferrell, 2007). Constitutively active kinases activate themselves by autophosphorylation in conformational loops as a response to external stimuli (Mori *et al.*, 2008). Some peptides of ChloroPhos1.0 are located in the transit peptide regions of the corresponding full length proteins. Due to this fact are incubations with stromal kinases also worthy to perform in order to identify protein phosphorylation dependent subcellular targeting. Based on newly MS-identified phosphorylation sites a second generation of the

peptide library could be designed, leading to the creation of "ChloroPhos2.0". Beyond such studies using peptide microarrays experiments on protein microarrays might be of interest for further investigations. Conformational aspects due to two-dimensional (2D) or 3D structures of the immobilized proteins, allow thereby the detection of distal docking sites.

The developed MS-phosphoproteomics pipeline might be of interest for further plant growth experiments for the used *Arabidopsis thaliana* genotypes, e.g. for light shift-, temperature-, compound- or osmotic stress conditions. These established protocols can be extended to other *Arabidopsis thaliana* genotypic backgrounds, like further silencing or t-DNA insertion lines. Also variations of this phosphoproteomics pipeline can be setup, e.g in order to address LTR targets of the nucleus, like components of the tetrapyrrole synthesis pathway (Baginsky, 2009; Crawford *et al.*, 2018; Park & Jung, 2017). Therefor other enrichment strategies might be used and coupled, for example a combination of a strong cation exchange chromatography (SCX) followed by an immobilized metal ion chromatography (IMAC) on the cellular proteome, as described in (Endler & Baginsky, 2011). Utilizing a variety of MS-fragmentation techniques might also lead to a deeper view into the phosphoproteome (Cox & Mann, 2008; Mann *et al.*, 2002). Especially the electron capture dissociation (ECD) and electron transfer dissociation (ETD) are promising applications for this purpose. Thereby the peptides are cleaved along their backbone while modifications and side chains remain intact during the fragmentation process (Baginsky, 2016; Syka *et al.*, 2004)

Appendix

Table 1 [A]: Results. Via ChloroPhos1.0 identified pCKII substrates with the respective AGI identifiers are listed. Functions of these substrates or their phosphorylations are annotated.

kinase	substrate			function in/ regulation of
	abbreviation	AGI	phosphopeptide	
pCKII (AT2G23070)	MSCS-like 3	AT1G58200	KAEKDEVSDDEATIE	osmotic stress response, ion transport
	EF1B	AT1G64510	FFEGGFGSDDDPTSP	plastid ribosome biogenesis, translational influence
	Alb3	AT2G28800	QSESEEG(S)DDEEEEA	LHC integration into thylakoids
			VEESQSESEEG(S)DDE	
	RCA	AT2G39730	RWRGLAYDTSDDQQD	activation of RuBisCo, onset of Calvin-Benson cycle
	Tac10	AT3G48500	KYEGKKLSELSDDED	function in PEP complex formation via PAP interaction
			GKKLSELSDDEDFDE	
	transcription activator	AT4G12610	DEEEGNVSDRGDEDE	DNA binding during transcription initiation from RNA polymerase II
	31RNP	AT4G24770	DASEGDVSEGDESEG	resistance against cold stress, RNA interaction for its stability and editing
	RH3	AT5G26742	AFKSLGLSDHDEYDL	plant growth, resistance against salt stress via splicing
	SSU	AT5G38410 AT5G38420 AT5G38430	LSYLPDLSDVELAKE	affects RuBisCo assembly, carbohydrate fixation
	cpHsc70-2	AT5G49910	DNGGDVIDADFTDSN	thermotolerance during germination, chaperone, transmembrane transport

Table 2 [B]: Results. Via comparative phosphoproteomics identified STN7 and STN8 substrates with the respective AGI identifiers are listed.

Functions of these substrates or their phosphorylations are annotated.

kinase	substrate			
	abbreviation	AGI	phosphopeptide	function in/ regulation of
STN7 (AT1G68830)	FNR	AT5G66190	LVY(T)DGGEIVK	ferredoxin-NADP+ oxidoreductase, terminal enzyme of linear electron flow in PS
	RPS7	ATCG00900 ATCG01240	VGGSTHQVPIEIG(S)TQGK	ribosomal assembly initiation, regulates its own gene expression
	ClpP3	AT1G66670	VoxMIHQPLGT_AGGK	degradation of misfolded proteins during embryogenesis
STN7 (AT1G68830) and STN8 (AT5G01920)	RNP29	AT3G53460	SSYGSGSG(S)GSGSGSGNR	transcript stability and integrity during chilling conditions
	RbcL	ATCG00490	LSGGDHIHAG(T)VVGK	RuBisCo assembly, carbon assimilation
STN8 (AT5G01920)	29-kDa ribonucleoprotein	AT2G37220	SSFGSSGSGYGGGGGSGAGSGNR	phosphorylation upon ABA stimulus, response to cold

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Halle (Saale), 31.07.2018

Anna Schönberg

Further Publications

Chapter 2 presents a list of my publications, which are integrated in this thesis.

Further publications, which were generated during my former scientific work at the department of evolutionary genetics, are listed below. Their content or methods are not elements of this thesis.

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5)   **Schönberg, Anna**; Theunert, Christoph; Li, Mingkun; Stoneking, Mark; Nasidze, Ivan (2011): High-throughput sequencing of complete human mtDNA genomes from the Caucasus and West Asia: high diversity and demographic inferences. European Journal of Human Genetics, 19 (9), 988-994.

6)   Li, Mingkun; **Schönberg, Anna**; Schroeder, Roland; Nasidze, Ivan; Stoneking, Mark (2010): Detecting heteroplasmy from high-throughput sequencing of complete human mitochondrial DNA genomes. The American Journal of Human Genetics, 87 (2), 237-249.

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich mich mit der vorliegenden Arbeit erstmals um die Erlangung des Doktorgrades bewerbe, die Arbeit selbstständig, ohne fremde Hilfe und nur unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt und die den benutzten Werken wörtlich und inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Halle(Saale), den 31.07.2018   Anna Schönberg